Physiological and genetic manipulation of adventitious rooting in Prunus spp

Thesis

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Thesis synopsis

Many species from economically important genera remain rooting recalcitrant, prohibiting the commercialisation of many species in forestry and horticulture, and hindering genetic improvement by conventional breeding or recombinant DNA technology, where vegetative propagation is often used to preserve the genetic fidelity of elite progeny. Two cherry species (*Prunus avium* and *P. padus*) were used as models in this study to investigate the physiological and genetic manipulation of adventitious rooting.

Mature trees are typically more difficult to propagate vegetatively than their juvenile counterparts. For some trees, micropropagation can circumvent certain effects of ageing and maturation, restoring shoot vigour and rooting, but the mechanism(s) involved have not been elucidated. During micropropagation, subculture interval was found not to be the predominant factor promoting the 'apparent rejuvenation' of mature *P. avium* tissue. 'Apparently rejuvenated' *ex vitro* and hedged (putatively) mature *P. avium* trees were treated with gibberellins predicted to have a range of structural related activities. GA_7 improved the rooting of cuttings from hedged (putatively) mature cherry, but not from *ex vitro* trees.

Methodology to regenerate adventitious shoots from *P. avium* leaf explants was developed, (putative) transgenic *P. padus* plants were produced by an *Agrobacterium tumefaciens* mediated strategy.

Auxin redistribution *in planta* is postulated to require a component of active transport; inhibition of the predominantly basipetal transport has profound effects on rooting. The putative function of the *Arabidopsis thaliana AtAUX1* gene is that of a cellular auxin influx carrier, possibly, as described by the chemiosmotic hypothesis. This thesis examined the hypothesis that transformation with the *AtAUX1* gene would enhance the delivery of the root-inducing signal to improve rooting of *P. padus*, a species which is rooting recalcitrant and more or less obligate on exogenous auxin for this process. However, all six, constitutively expressed, Cauliflower Mosaic Virus *35S* promoter driven, *35S::AtAUX1,* transgenic shoot lines had reduced rooting.

This thesis is dedicated to the memory of the late Christine Grant, and to Jacqueline Anne Grant whom both gave me the support and encouragement to pursue a scientific career; and my son Daniel Lachlan William Grant.

Hypothesis

The adventitious rooting potential of mature cherry is improved as a result of time spent *in vitro*, application of gibberellin to *ex vitro* and mature stockplants, prior to excising cuttings, and transgenesis, whereby constitutive ectopic expression of the *Arabidopsis thaliana AUX1* gene, under the regulation of the Cauliflower Mosaic Virus *35S* promoter is attained.

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Man is the interpreter of nature, science the right interpretation.

William Whewell 1794-1866

1. General Introduction

1.1 The Importance of Vegetative Propagation

Many woody perennials from economically important genera (e.g., *Quercus*, *Juglans* and *Pinus*) remain recalcitrant to adventitious rooting. Grafting of difficult-to-root scions onto rootstocks has been used to circumvent recalcitrant rooting, such as in the propagation of high value ornamental species and to establish seed orchards from mature elite trees. However, if feasible, in many circumstances this is inappropriate and/or economically prohibitive. Improved vegetative propagation may reduce the cost of existing plants (e.g., *Malus* and *Pyrus* dwarfing rootstocks) and/or provide opportunities for the commercialisation of new forestry (Ritchie, 1994) and ornamental (Howard, 1996) species, where adventitious rooting capacities can vary dramatically. For instance, the genus *Prunus* contains many species prized for their form and attractive blossom; the fastigiate *Prunus padus* described in this thesis has commercial potential as an ornamental but is considered difficult to root (Hammatt, 1993a).

Predominantly arable crops are grown from seed, as with a few exceptions (e.g., certain *Populus*, *Salix* and *Eucalyptus* species) are forest trees (Ritchie, 1994). However, the majority of arable crops have undergone extensive genetic improvement unlike most commercially important woody species. Consequently, seed for the propagation of many woody species is often of poor quality and/or unknown provenance (Nicoll, 1993). For a number of species (e.g., *Pinus radiata* and *Prunus avium*) seed orchards containing genetically superior trees have been developed to produce either open (OP) or control pollinated (CP) seed [Shellbourne (1987), Nicoll (1993)]. CP seed inevitably carries a cost premium and is often produced in insufficient quantities to meet demand (Ritchie, 1994). Moreover, inferior male pollen may contaminate OP orchards resulting in progeny with lower genetic gain and uniformity than those from CP produced seed. Vegetative propagation, via bulking (few copies of many genotypes) or cloning (many copies of an individual genotype) can improve productivity by increasing the number of progeny produced per seed.

Tree improvement programmes, whether by conventional breeding [Shellbourne (1987), Nicoll (1993)] or recombinant DNA technology [Walter (1998), Tzfira (1998)] often rely on vegetative propagation to preserve the genetic fidelity of elite progeny because high

heterozygosity and incompatibility mechanisms preclude propagation by seed. However, a recurring dilemma in many programmes is that phenotypic traits identifying a tree as superior are usually not evident until after the attainment of maturity and concomitant loss of adventitious rooting capacity. Possibly, the most cost effective method to propagate elite plants, while maintaining the high rooting capacity of the juvenile phase, is to establish hedges or stoolbeds from which conventional cuttings can be produced (Chapter *3*). Hence, poor rooting capacity can hinder the inception of such programmes and/or the subsequent evaluation and dissemination of superior plants.

Potentially, transgenesis (Chapter *6*) may by-pass many of the limitations associated with conventional tree breeding. However, if possible, propagation of transgenic trees by seed would require several generations of crossing to achieve genetic uniformity in progeny and take many years for most species. Frequently, production of transgenic plants by somatic embryogenesis is infeasible, therefore vegetative propagation is often essential to this technique and to *in vitro* propagation generally.

1.2 Adventitious roots: Form and Function

Vascular plants first became adapted to life on land during the late Silurian period, some 400 million years ago, and now fill a diverse range of habitats. Roots evolved for the acquisition of inorganic nutrients and water, within which the vascular system forms a continuum with the arial structures of these multicellular plants. They also provide anchorage, act as sites of biosynthesis (e.g., cytokinins and gibberellins) and storage (e.g., carbohydrates and nitrogenous substances).

1.2.1 Cellular organisation and patterning

Root tissues exhibit concentric radial symmetry with cells arranged longitudinally into columns (files) within which developmental maturity increases distal to the root meristem. Thus, a generalised root can be partitioned into four developmental zones: meristematic (cell division, determination and polarity establishment), elongation (cell expansion), specialisation (cell differentiation), lateral root development (*de novo* meristem initiation and organogenesis). This pattern is perpetuated by regular cell divisions within the meristem.

In certain species (e.g., *Arabidopsis thaliana*) the origin of cell files can be traced back to a single cell or specific subset of cells within the meristem, termed cell initials. These cells and

those of the quiescent centre (Clowes, 1956) form the promeristem (Scheres *et al.*, 1994). However, file progenitor cells may be less defined and files terminate within a broad zone at the root cap/body junction. Van den Berg *et al.* (1997) concluded, in *A. thaliana*, that the quiescent centre functioned to maintain initials by inhibiting differentiation in a process requiring cell-to-cell contact between the two. However, the generality of this model in species where contact between quiescent centre and initial cells is indirect is untested. Initials are postulated to form three primary tissues, protoderm, ground meristem and vascular procambium which differentiate into epidermis (trioblasts and atrichoblasts) and periderm, cortical (mainly parenchyma), and vascular tissues (phloem and xylem), respectively [Gunning (1982), Dolan *et al.* (1993), Baum and Rost (1996)].

Axis establishment and primary root architecture may be regulated by embryonic patterning genes [West and Harada (1993), Scheres *et al.* (1995), Benfey (1999)]. However, perpetuation of tissue patterning within the root is not considered to be controlled by lineage- or proliferation-dependent factors. Evidence from selective laser ablation (Van den Berg *et al.*, 1995), manipulation of mitotic cyclin genes (Doerner *et al.*, 1996) and radial patterning mutants [Torres-Ruiz and Jurgens (1994), Scheres *et al.* (1995), Traas *et al.* (1995)] is consistent with homoiogenetic induction, whereby mature cells of a particular fate promote immature cells to take on the same fate [Schiefelbein *et al.* (1997), Scheres and Berleth (1998)]. Cell-to-cell communication is an integral part of such regulation. Fluorescent marker dyes indicate tissue-specific symplastic connections, via plasmodesmata, within cortical parenchyma and epidermal tissues supporting a 'signal channeling' model (i.e., signal restriction within a pathway by regulatory or physical constraints) for cellular fate-determination within the continuity of each tissue layer (Scheres and Berleth, 1998). It has been speculated that auxin is the fate-determining signal.

1.2.2 Origin(s) of adventitious roots

Adventitious (from the Latin *adventicious*, meaning foreign) roots arise from locations other than within the radicle and the primary root. In certain genera (e.g., *Salix*, *Populus* and *Citrus*) latent adventitious root primordia, often located near stem nodes, may be present. However, adventitious roots usually develop by post-embryonic *de novo* meristem formation, as do lateral

roots. Nevertheless, primary, lateral (Malamy and Benfey, 1997) and adventitious roots [Ranjit *et al.* (1988), Harbage *et al.* (1993)] share morphological and physiology similarities.

Histological analyses of cuttings derived from micropropagated [e.g., *Prunus avium* and *P. avium* x *P. pseudocerasus* (cv. Colt), Ranjit *et al.* (1988); *Malus domestica*, Hicks (1987), Harbage *et al.* (1993); *Castanea sativa*, Ballester *et al.* (1999)] and conventionally propagated donor plants [e.g., *Pinus contorta*, Gronroos and Von Arnold (1987); *Pinus strobus*, Goldfarb *et al.* (1998)] suggest *de novo* root primordium initiation frequently begins in or near tissues of the pericycle, ray and vascular parenchyma or their cambial precursors. For instance, Ranjit *et al.* (1988) observed root meristem initiation within Colt phloem parenchyma, external to the cambium and cambial region, and within the cambium of seedling and mature *P. avium* shoots. The latter of which had been co-cultivated with Colt shoots and consecutively subcultured *in vitro* for several months, therefore described as 'invigorated' by the authors. These results suggest that derivatives of cambium cells which normally function as a secondary meristem acquired the developmental characteristics of a primary meristem. In a number of species adventitious roots develop indirectly via a callus phase [e.g., *Pinus radiata*, Cameron and Thomson (1969); *Pinus taeda*, Hamann (1998)]. This developmental pattern is slower than that of direct formation and is often associated with difficult-to-root species. For some species, a shift in the pattern of root initiation from direct to indirect correlates with maturation [e.g., *Pinus strobus*, Goldfarb *et al.* (1998); *Hedera helix*, Geneve *et al.* (1988)], but not in others [e.g., *P. taeda*; Hamann (1998)]. Exogenously applied auxin can promote a change in the pattern of development from indirect to direct [e.g., *P. contorta*, Gronroos and Von Arnold (1987); *Pinus sylvestris*, Flygh *et al.* (1993); *P. taeda*, Hamann (1998)].

The formation and development of lateral root primordia has been extensively studied in *A. thaliana* and a similar development process may be applicable to that of adventitious roots. However, *A. thaliana* roots are anatomically simpler than those of many species. Root primordia in *A. thaliana* are often initiated opposite a protoxylem pole that connects with the primary root xylem (Malamy and Benfey, 1997). Primordia form within the pericyclic parenchyma cells adjacent to the vasculature. Initiation at a single cell, or group of cells, begins with a series of periclinal then anticlinal divisions to produce a four layered meristematic dome, which protrudes into the cortex. The root primordium emerges through the cortex and epidermis of the parent

root, followed by root cap development and quiescent centre formation. The cell wall of the emerging root may be reinforced by the product of a hydroxyproline abundant glycoprotein gene [HRGPnt3; Keller and Lamb (1989)]. Emergence is driven principally by cell expansion at the primordium base since the meristem is inactive until it has cleared the parent root.

Development of the emergent root results in transient root hair and Casparian band(s) production (Peterson, 1988). Secondary growth occurs in many dicotyledons. Within woody perennials, pericycle vascular cambium initiation begins and proliferates by arcing laterally to eventually envelop the xylem and produce secondary xylem endarch and secondary phloem exarch.

1.3 Developmental Phase

Progression through the life cycle of a woody plant (ontogenesis) can be partitioned into a series of developmental episodes or phases based on distinctive morphological or physiological characteristics. Thus, juvenile (embryonic and post-embryonic), mature vegetative and reproductive (sexually competent) phases can be distinguished within this developmental continuum [Hackett (1985), Poethig (1990)].

The developmental phase of a propagule may be a significant factor acting upon its proficiency to initiate adventitious roots (Murray *et al.*, 1994). Thus, mature woody perennials are, typically, more difficult to propagate vegetatively than their juvenile counterparts [Hackett (1985, 1988), Poethig (1990), Greenwood and Hutchison (1993), Greenwood (1995)]. For some trees, micropropagation can circumvent certain effects of ageing and maturation, restoring shoot vigour and adventitious rooting capacity (Chapter *3*). However, little is known about the underlying mechanism(s) promoting these changes.

1.3.1 Maturation and chronological ageing

Woody plants are perennial, static and autotrophic. Their sustained development and ability to elude localised resource depletion requires continual expansion of the surface area devoted to the uptake and capture of nutrients and sunlight, and the cycling of nutrients internally. However, within the tree competition for nutrients may become limiting on growth because of increased size and complexity (Moorby and Wareing, 1963). Thus, competition for resources within the tree may promote a progressive decline in annual growth increment (vigour) and geotropic

responsiveness [Moorby and Wareing (1963), Hackett (1985), Greenwood and Hutchison (1993)]. Moorby and Wareing (1963) proposed such a hypothesis to model chronological or physiological ageing.

Concurrent with chronological ageing are further morphological, physiological and biochemical changes (e.g., phyllotaxy and reduced adventitious rooting competence) which are associated with the progressive acquisition of sexual reproductive competence, and termed maturation or ontogenic ageing [Wareing (1959), and Fortainier and Jonkers (1976), respectively]. Significantly, the gradual loss of juvenile vigour (e.g., height and diameter incremental growth) during chronological ageing can sometimes be reversed by grafting onto rootstocks or pruning (i.e., removal of aged and complex structure), in contrast to the frequently persistent nature of maturational traits, e.g., flowering competence (Hackett, 1985). However, scions of the same size, from trees of different ages, exhibit differences in growth rate, indicating that such a division may be too simplistic and loss of vigour can be attributed to ageing, maturation, or both (Greenwood and Hutchison, 1993). Additionally, the processes of chronological/physiological and maturational/ontogenic ageing may produce disparities in the developmental phases of structures within the tree.

1.3.2 Coexistence of developmental phases

Typically, structures (e.g., inflorescence and vegetative apices) representing different developmental phases appear to coexist stably along the shoot axis, thereby suggesting a holistic assignment of developmental phase is required. Models have been proposed [Hackett (1985), Poethig (1990)] in which the phase identity of structures (e.g., lateral buds or leaf morphology) laid down while the meristem occupied a specific development phase, which it may no longer maintain, is preserved along the shoot axis (heteroblasty). Hence, the chronological age of structures on a shoot decreases acropetally, in contrast to their increasing maturation (topophysis). Therefore, basal structures may be more likely to retain juvenile (e.g., competence for vegetative propagation) characteristics as the plant ages chronologically (Greenwood and Hutchison, 1993). However, such models suggest it may be more accurate to view developmental phase at the level of the shoot apex, and therefore appropriate to consider a woody perennial as a colony of semi-autonomous meristems (Trewavas, 1983).

1.3.3 Control of developmental phase

Phase-specific traits can be retained after grafting mature meristems onto juvenile rootstocks, e.g., *Citrus* (Navarro, 1975) or ivy (Hackett, 1985), implying that they may exist in either mature or juvenile states. However, determination of the meristem to a particular maturational phase does not necessarily mean pre-determination at the cellular level to the formation of a specific cell type or tissue. Several models have been proposed to explain maturation based on the control of cellular phase determination being either intrinsic or extrinsic to the apical meristem. For instance, Poethig (1990) cites evidence (mainly from herbaceous plants) for factors that are extrinsic to the meristem initiating maturational processes, with competence to respond to these factors and phase stability regulated by factors within the meristem. Poethig's model describes phasic development as being regulated in an additive manner by a series of independent programmes, a view based upon the intermediate morphology of structures during plant development. However, Greenwood and Hutchison (1993) conclude from scion rootstock interactions that intrinsic changes to the meristem (e.g., a critical number of cell divisions) may also be required for the initiation of maturation. Alternatively, maturation may be initiated by a number of biological (juvenile/mature state) switches (Hackett, 1992), acting either in parallel or series. Greenwood (1987) simplifies this hypothesis to a single biological switch (i.e., the mosaic meristem model) where the proportion of mature phase cells within the apical meristem gradually increases and results in expression of maturational traits.

There are several speculative mechanisms for the stable maintenance of developmental phase based on epigenetic (i.e., mitotically and/or meiotically heritable changes in the function of a gene without changes to the DNA sequence) changes to the genome, e.g., DNA methylation, alteration of chromatin structure or limited gene rearrangement [Poethig (1990), Greenwood and Hutchison (1993)]. An alternative, non-genetic, mechanism proposed by Greenwood and Hutchison (1993) is based upon the expression of cellular maturation resulting from differences in the physical availability of membrane-bound receptors if, with time, they became increasingly internalised within the cell.

1.3.4 Rejuvenation

Phase change appears to be a reversible process. Rejuvenation from somatic cells (i.e., the return to sexually incompetent vegetative growth) occurs as a consequence of sexual, or as in *Citrus,* adventitious embryo formation from the diploid nucellus in apomictic reproduction. Whether this results from true *in situ* rejuvenation of mature cells or by activation of vestigial juvenile cells that never underwent maturation is unknown. Additionally, shoots displaying some level of rejuvenated character may occur naturally (e.g., root suckering or epicormic shoots), or may be induced by grafting onto rootstocks, pruning, treatment with growth regulators or by micropropagation. Of particular significance to this thesis are how the latter two methods affect adventitious rooting potential (Chapters *3* and *4*).

1.4 Auxin: A root-inducing signal

Endogenous auxin influences numerous physiological and developmental processes, including shoot apical dominance, vascular differentiation, photo- and gravitropism, bilateral symmetry, lateral and adventitious root development (Davies, 1995). Auxin may regulate such processes exclusively, in association with, or counter to other endogenous growth regulators (Coenen and Lomax, 1997). There is substantial evidence illustrating the efficacy of exogenously applied auxin as a root-inducing stimulus (Haissig and Davis, 1994) but much of the detail on the mechanism(s) involved and the interactions between applied (exogenous) and endogenous auxin remain to be elucidated.

1.4.1 Endogenous auxin(s)

Several endogenous auxins have been identified in higher plants e.g., indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and 4-chloroindole-3-acetic-acid (4-Cl-IAA) [Epstein and Ludwig-Muller (1993), Bandurski *et al.* (1995), Reinecke (1999)]. Gas chromatography mass spectrometry (GC-MS) analysis implies that young pea (*Pisum sativium*) seedlings contain these three auxins (Schneider *et al.* 1985). Additionally, IBA has been identified in a number of species by GS-MS (e.g., *A. thaliana*, *Zea mays*, and *Daucus corota*). *In planta*, IBA biosynthesis from IAA may occur by a chain elongation reaction analogous to the primary steps in fatty acid biosynthesis. Conjugation and deconjugation of IBA has been demonstrated, as has conversion of IBA to IAA, but whether IBA is active directly *in vivo* or exerts auxin activity via conversion to IAA is undetermined [Epstein and Ludwig-Muller (1993), and Bartel (1997)]. Thus far, 4-Cl-IAA has

been detected, by GC-MS, principally within various species of the Vicieae tribe of Fabaceae including *Vicia faba* and *Pisum sativum*, but identification in *Pinus sylvestris*, which belongs to a different plant subdivision (Gymnospermae), suggests a wider Kingdom distribution. The biological role of 4-Cl-IAA is uncertain, but this potent auxin (relative to IAA in several bioassays) may be involved in regulation of certain stages of development (e.g., seed and fruit growth) or in the induction of senescence (Reinecke, 1999). Excepting perhaps IAA, little is presently known about the physiological relevance of these auxins. Furthermore, although IAA is considered the most abundant endogenous auxin *in planta*, the pathway(s) of IAA biosynthesis, localisation of gene expression, and activity of enzymes involved have not been unequivocally established *in vivo* [Normanly (1995, 1997), Bartel (1997)]. However, the concentration of biologically active (non-conjugated) free IAA in any cell or tissue depends on the interplay between the processes of biosynthesis, conjugation, deconjugation, catabolism, inter auxin conversion, compartmentalisation and transport flux.

1.4.2 Site(s) of de novo biosynthesis

De novo IAA biosynthesis is postulated to occur primarily at, or near the shoot apex with subsequent translocation throughout the plant. Consistent with this model, encircling *Pinus sylvestris* stems below the shoot apex with the polar auxin transport inhibitor *N*-1-naphthylphthalamic acid (NPA) resulted in localised IAA depletion below the site of application (Sundberg *et al.*, 1994), and NPA applied to the shoot-root junction of *A. thaliana* seedlings inhibited lateral root development (Reed *et al.*, 1998). Furthermore, in *Populus* the highest concentration of endogenous IAA has been localised to cambial meristem and derivative tissues implicated in basipetal auxin transport (Tuominen *et al.*, 1997). Leaves have been proposed as a potentially significant site of IAA biosynthesis. Cambridge and Morris (1996) demonstrated that radiolabelled IAA could be transported from mature pea (*Pisum sativum*) leaves to the immature tissues of the shoot apex, via the phloem, and subsequently entered the basipetal transport steam. However, Sundberg and Little (1987) concluded the level of IAA entering the basipetal transport stream of 1-year-old balsam fir shoots (*Abies balsamea*) was unaffected by their defoliation.

Evidence suggestive of additional *de novo* IAA biosynthesis potential other than at or near the shoot apices has been shown: the labelled to unlabelled IAA ratio of stem tissues of decapitated *Pinus sylvesteris* 4-year-old saplings complemented with [$^{13}C_6$]-IAA (Sundberg and Uggla, 1998), and in isolated *A. thaliana* roots supplemented with [$^{2}H_5$-L-tryptophan (Muller *et al.*, 1988). Speculatively, such results are interesting since they may begin to explain the enigma of how apically supplied auxin can modulate root development, especially in trees, where the distance between the two may be great, and basipetal IAA transport rates are relatively slow, approximately 10-20 mm h^{-1} in a wide range of species (Lomax *et al.*, 1995). However, the study by Reed *et al.* (1998) suggests a requirement for auxin derived from the shoot, at least in some aspects of root development, within intact *A. thaliana* seedlings. Thus, the physiological relevance of IAA biosynthetic capacity in *A. thaliana* roots, especially where the shoot-to-root junction has been circumvented, may be equivocal. Additionally, a correlation between the presence of the shoot apex, and basipetal auxin transport from it, with the rooting potential of rooting recalcitrant species has been shown (Section *1.4.9*). Overall, such results suggest further studies are required to determine fully both the locations(s) and relative importance of all putative IAA biosynthesis sites to auxin homeostasis *in planta*.

1.4.3 Biosynthesis pathway(s)

De novo IAA biosynthesis suggests the fabrication of a heterocyclic indole ring from non-aromatic precursors. *In planta*, this implies some deviation from the shikimic acid pathway, which is the only known source of all naturally occurring aromatic amino acids (Bandurski *et al.*, 1995). Chorismate, the common branch point intermediate in the biosynthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan is synthesised initially by a condensation reaction between phosphoenolpyruvate (a glycolytic intermediate) and erythrose 4-phosphate (a pentose intermediate) and proceeds via the intermediate shikinmate. Evidence suggests IAA biosynthesis may occur in *planta* via either presumptive IAA precursor tryptophan-dependent or tryptophan-independent pathways [Normanly *et al.*, (1995), Bartel (1997)].

Tryptophan is synthesised from chorismate via the pathway intermediates anthranilate, *N*-5-phosphoribosyl-anthranilate, 1-(o-carboxyphenylamino)-1-deoxyribose 5-phosphate, indole-3-glycerol phosphate (IGP) and finally indole (Bandurski *et al.*, 1995). Several non-aseptic

studies have shown tryptophan can be converted to IAA by plant extracts *in vitro* (Nonhebel *et al.*, 1993). However, as Normanly (1995) cautions, this conversion can occur non-enzymatically or because of bacterial contamination. Nevertheless, germinating *Phaseolus vulgaris* seedlings (Bialek *et al.*, 1992) and *A. thaliana* roots (Muller *et al.*, 1998) are competent to synthesise IAA from labelled $[^2H]_5$-L-tryptophan under reportedly aseptic, controlled conditions. The precise detail of the pathway(s) producing this conversion is still speculative but several independent pathways with either indole-3-acetamide (IAM), indole-3-acetonitrile (IAN) and indole-3-pyruvic acid (IPA) as intermediates are possible (Bartel, 1997).

Michalczuk *et al.* (1992) observed both tryptophan-dependent and tryptophan-independent IAA biosynthesis in *D. carota* and several studies strongly suggest tryptophan-independent auxin biosynthesis can occur in other species. *A. thaliana* mutants with lesions blocking biosynthesis before IGP [*trp1-1*; Last and Fink (1988)] have an auxin deficient phenotype, whereas, those blocked between IGP and tryptophan [*trp2* and *trp3*; Normanly *et al.* (1993)] contain wild-type free IAA, but elevated IAA conjugates and IAN levels. Nitrilases capable of converting IAN to IAA have been identified and cloned from *A. thaliana* (Bartel and Fink, 1994). Thus, a branch point may lie between IGP and tryptophan in which IAN may act as an intermediate for IAA biosynthesis. Consistent with this view, the tryptophan auxotrophic mutant (orange pericarp, *orp*) of *Z. mays*, which has a lesion preventing tryptophan synthase-B production, accumulates a higher concentration of IAA conjugates, shows deuterium oxide labelling enrichment of IAA but not tryptophan, and [^{15}N] anthranilate labels only IAA (Wright *et al.*, 1991).

1.4.4 Conjugation, deconjugation and catabolism

Most IAA *in planta* is found conjugated, predominantly via amide bonds to amino acids, peptides and proteins, or by ester bonds to sugars or myoinositol. IAA overproducing mutants [e.g., *sur1*, *Boerjan et al.* (1996); King *et al.* (1995); and *fass*, Fisher *et al.*, (1996)] have substantially elevated conjugate levels relative to free IAA implying conjugation may have a dominant role in the removal of excess IAA. However, IAA conjugation hydrolysing activity has been demonstrated. Thus, conjugation may act as a reservoir into which free IAA can be shunted or released to maintain auxin homeostasis. Additionally, conjugation moieties may function to target

IAA to a particular location: perhaps as a function of their degree of lipophilicity and/or protect against peroxidative degradation.

Evidence suggests the primary catabolic route is via oxidation to oxinodole-3-acetic acid and glycosylation into a biologically deactivated form (Normanly *et al.*, 1993).

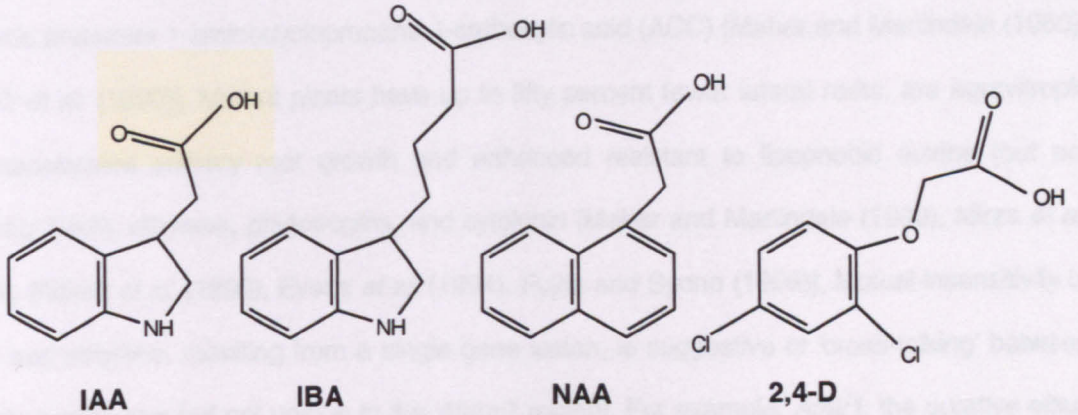
1.4.5 The chemiosmotic hypothesis of auxin transport

Endogenous and exogenously applied auxin(s) may be translocated *in planta* by passive (mass flow or diffusion) or active (carrier-mediated) processes. Within the phloem, the cytoplasmic continuity between cells mediates transport intra-cellularly, whereas transport is effectively extra-cellular within xylem and reliant upon the motive force of the transpiration stream. In dicots, active auxin transport is postulated to occur primarily in tissues of the vascular (mainly phloem) and the extra-vascular procambium, cambium and differentiating phloem vascular elements (Lomax *et al.*, 1995).

The molecular mechanism(s) promoting a predominantly basipetal auxin flux *in planta* mainly remain to be elucidated but molecular and genetic research are providing significant insights. The chemiosmotic hypothesis [Rubery and Sheldrake (1974), Raven (1975)] of polar auxin transport predominates. This model proposes that net basipetal auxin flux is driven by proton motive force and carrier-mediated with symmetric influx and asymmetric, basally localised, efflux carrier distributions within the plasma-membrane of transporting cells. There is much evidence supporting this model. Radiolabelled auxin (^{14}C-IAA) uptake into sealed, cytoplasmic face out, microsomal vesicles of *Cucurbita pepo*, which mimic *in planta* trans-plasma-membrane pH and electrical gradient, are sensitive to inhibition by phytotropins, metabolic poisons, dissipation of the transmembrane electrical potential, and auxin saturation [Goldsmith (1977), Lomax *et al.* (1985), Sabater and Rubery (1987)]. These effects are consistent with auxin transport inhibitor studies on whole plants or organs [e.g., Fujita and Syono (1996), Reed *et al.* (1998)], an electrogenic requirement, and the specificity and saturability associated with protein mediated carrier(s). That carrier-mediated auxin uptake is dependent on proton motive force rather than pH gradient has been demonstrated: vesicles loaded with KCl in a low K^+ medium rapidly accumulate ^{14}C-IAA when a K^+ specific ionophore (valinomycin) is added but a K^+/H^+ ionophore (Nigericin) or protonophore (FCCP) reduced auxin flux to background levels (Lomax *et al.*, 1985).

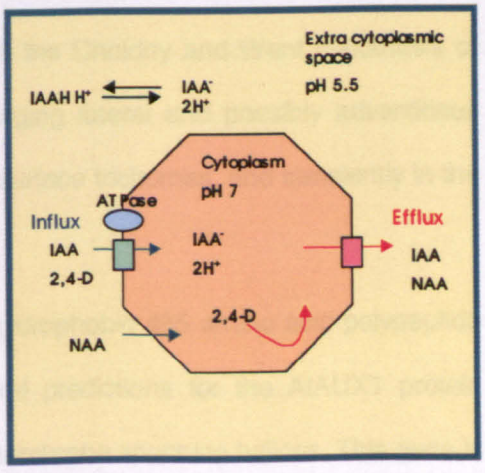
Auxins are weak acids (e.g., IAA pk_a 4.7) and contain a common polarised, unsaturated, carboxylic acid functional group (Figure *1a*).

Figure 1a. Four frequently used auxins, indicating the carboxylic acid functional group (yellow shaded box)



Auxins may become more protonated in the acidic (*ca.* pH 5.5) extra-cytoplasmic apoplast than within the relatively basic (*ca.* pH 7) cytoplasm (symplast). Protonated auxins, may transverse the plasma membrane directly or via a saturable influx carrier (Figure *1b*), powered as a proton-driven symporter. Uptake analyses of radiolabelled probes into microsomal vesicles and membrane pH potential suggest a $2H^+/IAA^-$ symport may be more probable than $IAAH/H^+$ (Sabater and Rubery, 1987). Within the cytoplasm, deprotonation of auxin occurs and the undissociated auxin anion is effectively trapped within the cell because of its lowered membrane permeability. Efflux of the auxin anion is postulated to take place via a saturable efflux carrier. The substrate affinity of different auxins for these carriers varies, as does auxin plasma-membrane permeability. Thus, 1-naphthalene acetic acid (NAA) can enter the cell by passive diffusion, whereas IAA and 2,4-dichlorophenoxyacetic acid (2,4-D) are actively influxed. Intracellular auxin concentration is regulated by the efflux carrier for NAA, whereas 2,4-D is regulated by influx, and IAA by both mechanisms (Delbarre, 1996). Thus far, the mechanism by which IBA is transported across the plasma membrane is unknown.

Figure 1b. Putative model of auxin flux across the plasma-membrane



1.4.6 AUX1: The putative auxin influx carrier

Molecular genetic approaches have begun to identify and characterise components of the auxin transport mechanism. Recessive mutants, allelic for defects in the *Arabidopsis thaliana AUX1* (*AtAUX1*) gene, were isolated by screening mutagenised *A. thaliana* seedling populations for disrupted gravitropism or root growth on normally inhibitory concentrations of IAA, 2,4-D or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) [Maher and Martindale (1980), Pickett *et al.* (1990)]. Mutant plants have up to fifty percent fewer lateral roots, are agravitropic with accelerated primary root growth and enhanced resistant to lipophobic auxins (but not lipophilic NAA), ethylene, phytotropins, and cytokinin [Maher and Martindale (1980), Mirza *et al.* (1984), Pickett *et al.* (1990), Evens *et al.* (1994), Fujita and Syono (1996)]. Mutual insensitivity to auxin and ethylene, resulting from a single gene lesion, is suggestive of 'cross-talking' between signalling pathways but not unique to the *Ataux1* mutant. For example, *Ateir1*, the putative efflux carrier, is resistant to both (Lusching *et al.*, 1998) and *Ataxr2* mutatants are resistance to auxin, ethylene and abscisic acid (Wilson *et al.*, 1990). This is consistent with the hypothesis that ethylene may act via auxin.

Promoter β-glucuronidase fusions and whole-mount *in situ* hybridisation [Bennett *et al.* (1996), Ward and Bennett (1996)] imply that the *AtAUX1* gene is spatially expressed predominantly in root tip epidermal cells, consistent with the Choldny and Went hypothesis of gravitropism (Rosen *et al.*, 1999), and regions of emerging lateral and possibly adventitious roots. To a lesser extent, expression also occurs in leaf surface trichomes, and transiently in the shoot apical meristem.

The *AtAUX1* gene (4.46 kb) encodes a highly hydrophobic 485 amino acid polypeptide with a predicted molecular mass of 54.1 kD. Structural predictions for the AtAUX1 protein suggest a molecule with between ten and twelve trans-membrane spanning helices. This gene is homologous with a family of fungal (*Neurospora*) and *Nicotiana* sequences known to encode functional amino acid permeases (Bennett *et al.*, 1996) with greatest similarity to *Arabidopsis* AAP1. Mechanistically, plant amino acid permeases may function as proton-driven symporters [Sabater and Rubery (1987), Boorer (1996), Boorer and Fischer (1997)]. These similarities and the structural resemblance of IAA to the amino acid tryptophan led Bennett *et al.* (1996) to postulate that the AtAUX1 protein may be an auxin influx carrier, as proposed in the

chemiosmotic hypothesis of polar auxin transport (Section *1.4.5*). Consistent with an auxin influx role, auxin induced rooting responses take twice as long for *Aaux1* mutant than wild-type plants (Evans *et al.*, 1994), but this is conditional upon the assumption that at least one site for auxin perception is intracellular. Yamamoto and Yamamoto (1998) observed that the gravitropic response could be restored in *Aaux1* roots by NAA, but not IAA or 2,4-D. This is compatible with differential uptake of auxins by tobacco suspension-cultured cells reported by Delbarre *et al.* (1996). No significant anatomical difference between wild-type and *Aaux1* mutant plants in root ultrastructure (Olsen *et al.*, 1984) imply that the *AtAUX1* gene is not required for root differentiation and development. Likewise, deficiency of any major arial phenotype in the mutant implies a role for *AtAUX1* predominantly within the root. This view is supported where hypocotyl etiolation, leaf chlorophyll loss and peroxidase activity were equally affected by ethylene in both mutant and wild-type *Arabidopsis* plants (Pickett *et al.*, 1990).

The *AtAUX1* gene is a member of a small family of closely related genes, all or some of which may function in auxin uptake (Bennett *et al.*, 1996). Orthologues of *AtAUX1* may perform similar functions but have a different temporal and/or spatial patterns of expression. Conceivably, loss of function of other members of this group may result in further reaching consequences. Related, highly conserved, homologues of *AtAUX1* have been identified in several higher plant species (e.g., cherry, *Prunus avium*; tomato, *Lycopersicon esculentum*; maize, *Zea mays*; and rice, *Oryza sativa*). Hence, information gained as to the biological function of *AtAUX1* may be applicable to other plant systems.

1.4.7 The putative auxin efflux carrier

A gene encoding a presumptive efflux carrier has been isolated by four groups [Lushnig *et al.* (1998), Muller *et al.* (1998), Utsuno *et al.* (1998), Chen *et al.* (1998)]. The gene has multiple names [*AGR* and *WAV6*, Bell and Maher (1990), Okada and Shimura (1990); *EIR1*, Roman *et al.* (1995) and *PIN2*, Muller *et al.* (1998)] since reverse genetics of the mutants were carried out independently. The gene encodes a 69 kDa protein, predicted to have ten transmembrane domains with two groups of five domains at each terminus separated by a hydrophobic rich region. The protein has 35-40 percent similarity with bacterial proteins known to function in transport. Supporting evidence for this gene encoding the efflux carrier includes:

- EIR expressing yeast cells are more resistant to toxic IAA derivatives (flouroindoles).

- *eir1* mutants are not resistant to exogenous auxin, a characteristic that would more likely be associated with an influx defect.
- *alf1* mutants have elevated auxin levels, increased lateral rooting and decreased root elongation, whereas in *alf1/eir1* double mutants this phenotype elevated.
- AGR expressing yeast cells release IAA more rapidly than wild-type cells, suggesting AGR facilitates auxin efflux.
- *A. thaliana agr* mutant plant roots grow into medium with IAA/NAA, whereas wild-type roots stay on the surface. 2,4-D does not elicit this response in either wild-type or mutant plants (Ustsuno *et al.*, 1998).
- *agr1* seedling root tips pre-loaded with radio-labelled auxin retain it longer than wild-type (Chen *et al.*, 1998).

Jaccobs and Gilbert (1983) demonstrated the basal localisation of a putative efflux carrier using an immunological approach: monoclonal antibodies, which prevented the binding of the phytotropin *N*-1-naphthylphthalamic acid (NPA) to microsomal membrane proteins, bound specifically to the basal plasma-membrane of pea stem cells. Furthermore, the *Arabidopsis thaliana* AtPIN1 (Okada *et al.*, 1991), and AtPIN2 (Muller *et al.*,1995) proteins, members of a multigene family [thus far, 14 have been identified (K. Palme, Pers. Comms., 2000)] of putative efflux carriers have been localised to the basal plasma-membrane of cells involved in auxin transport within the stem (*AtPIN1*) and root (*AtPIN2*), respectively.

1.4.8 Perception and Signal transduction

Little of substance is known about the pathways of auxin signal transduction. However a model of gene activation initiated by auxin has become apparent.

Dissection of the pathways associated with auxin biosynthesis, metabolism, signal transduction and perception would reveal many potential sites where mutations may effect lateral and/or adventitious rooting competence [e.g., synthesis - *sur1*, King *et al.* (1995); conjugation - fass, Torres-Ruiz and Jurgens (1994); deconjugation - *1lr1*, Bartel and Flink (1995), transport - *Ataux1*, Bennett *et al.* (1996); signal perception - *axr1*, Leyser *et al.* (1993) and transduction, see reviews by Hobbie and Estelle (1994), Walden and Lubenow (1996), and Leyser (1997)].

However, thus far, many of the mutants identified have been associated with lateral root initiation and development. Thus, it is pertinent to question their relevance to an investigation of the molecular and genetic control of adventitious rooting.

1.4.9 Auxin and the shoot apex

Observations by Marks (1996) suggest endogenous basipetal transported auxin from the shoot apex may be sufficient to mediate rooting in easy-to-root species (e.g., *Betula pendula*) but not recalcitrant species (e.g., *Daphne cneorum*). In a number of species [e.g., *Helianthus annuus*, Liu and Read (1992); *Daphne cneorum* and *Quercus robur*, Marks (1996); *Pisum sativum*, Koukourikou-Petridou and Bangerth (1997); *Rhododendron obtusum*, Chen *et al.* (1997)] excision of the shoot apex or inhibition of basipetal auxin transport from the apex with the phytotropins NPA [*Rumex* spp., Visser *et al.* (1995); *Pinus taeda*, Greenwood and Weir (1995)] or 2,3,5-triiodobenzoic acid (TIBA) [Liu and Read (1992), Marks (1996)] reduced adventitious rooting capacity. Dependency on the presence of a shoot apex, and number of buds (and leaves) per shoot, could be correlated with potential for recalcitrant rooting [Marks, (1996), Marks and Simpson (2000)]. De Klerk *et al.* (1995) concluded similar requirements with the rooting of *Malus* 'Jork 9' stem slices. Partial re-establishment of rooting potential could be achieved by applying auxin to the distal regions of decapitated *Helianthus annuus* hypocotyls (Liu and Read, 1992) and *Betula pendula* shoots, or in rooting recalcitrant *Betula* internodal sections by substitution with a bud. However, this did not occur with rooting recalcitrant *Daphne cneorum* [Marks (1996), Marks and Simpson (2000)]. That rooting capacity could only be partially restored by exogenous auxin may also indicate a prerequisite for an endogenous source. Marks and Simpson (2000) propose a rooting model conditional upon acropetal transport of exogenous auxin within the phloem to the shoot apex where it is transferred to the basipetal polar auxin transport stream. Thus, endogenous auxin may have a role in controlling the rooting response directly or by facilitating an interaction with exogenously applied auxin.

1.5 Molecular Genetic Control of Adventitious Rooting

1.5.1 Cellular competence

Production of autonomous plants by vegetative propagation may ultimately depend on the propagule's inherent capacity (cellular competence) to respond to specific root-inducing stimuli and thereby become determined (i.e., committed), even upon removal of the stimuli, to a certain

developmental fate and differentiate to form an adventitious root (Mohnen, 1994). *Convolvulus arvensis* leaf explants require auxin for both attaining adventitious rooting competence and determination [Christianson and Warnick (1985), Warnick (1992)], similarly so did *Nicotiana tobacum* thin cell layer explants, excised from floral branches (Mohnen, 1994). This suggests that, for some species at least, competence is not inherent in tissues taken from the mother-plant. Christianson and Warnick, (1985) and Warnick (1992) concluded that *Convolvulus* leaves took about 72 hours to become competent and that failure of some genotypes to root resulted from inability to acquire competence. Nevertheless, the molecular basis for attainment of cellular competence and the induction of the determined state has not been elucidated (Mohnen, 1994).

Wareing and Graham (1984) postulated that rooting competence may be influenced by differences in the level of expression of genes encoding receptors (or the cellular turnover of receptors) for the root-inducing stimuli. Thus, maturational effects on adventitious rooting capacity may result from modified receptor configuration. Comparative studies of debladed petioles from mature and juvenile phase *Hedera helix* revealed phase-dependent differences in adventitious rooting formation (Geneve, 1988). Wounding alone induced no response from ivy in either phase, but with auxin, callus formed on both suggesting propagules in either phase were capable of perceiving the root inducing signal but only juvenile propagules were competent to respond. Histological investigation suggests that mature phase petioles lacked co-ordination in controlling the location and orientation of divisions within the cortical parenchyma and were therefore unable to initiate root primodia (Geneve, 1988). Woo (1992) found the adventitious rooting incompetent state of mature phase ivy to be non-absolute and expression of a gene encoding a proline-rich protein (*PRP*) could be correlated with excision of cuttings and inversely related to competence for adventitious root formation. He postulated that the significantly higher levels of this *PRP* in mature phase cuttings caused the disruption of root primordia formation in ivy.

Improved knowledge of factors influencing and/or controlling development phase, and/or the mechanism(s) of perception, transduction and response(s) to root-inducing stimuli, and how these constituents interact *in planta* with the state of competence may provide opportunities for overcoming rooting recalcitrance. Physiological and biochemical (post-translational) analysis can identify factors correlated with adventitious rooting competence, but will not necessarily elucidate

the underlying mechanism(s) of control. Ultimately to elucidate these mechanisms requires a reductionist approach whereby the effects on adventitious root formation of the manipulation of the gene(s) or gene families involved can be attempted. Thus, a molecular genetic approach provides tools for testing the hypothesis that specific genes have direct effects on rooting competence, and views adventitious rooting recalcitrance as a component of a problematical plant development pathway.

1.5.2 Genes and rooting

The application of molecular genetics to the investigation of adventitious rooting competence is based upon the hypothesis that rhizogenesis has a genetic component, and that specific genes can have a direct effect on this process. Therefore, adventitious rooting can be viewed as a more manageable, specific subset of all the developmental processes that occur *in planta* (Haissig, 1992). Zobel (1986) suggests the number of genes controlling this developmental process may be relatively large. However, evidence suggest that at least some gene interactions can be studied independently from others (Greenwood, 1993). For example, in tomato four to six loci were found to control fruit mass, solids content and pH in a additive, as opposed to a co-operative manner (Paterson *et al.*, 1988), or mutations in single genes affecting lateral/adventitious rooting (reviewed in Leyser, 1997). Riemenschneider (1994) proposes that a quantitative threshold model (based upon the additive effect of several genes) be adopted as the default for researching the genetic control of adventitious rooting rather than qualitative (single gene with major effect), since he concludes insufficient evidence exists to assume the latter. In such a model, he views rooting as a discontinuous state (rooting/non-rooting). Thus, rooting is attained by the interaction of a root-inducing stimulus (e.g., auxin/wounding) with some form of cellular receptor that initiate the transduction of the rooting signal through one or more genetically controlled biochemical pathway(s) of undetermined complexity. Rooting occurs when the root-inducing stimuli through this transduction pathway exceeds (a) given threshold value(s). If attainment of the threshold value (i.e., rooting competence) requires the additive effects of many genes, successful modification to exceed this threshold is more probable for a modification to a single enzyme pathway, or a regulatory locus that would simultaneously modify the expression of many loci (Riemenschneider, 1994). For instance, that at least one receptor site for auxin perception may be intracellular, is suggested by the *Ataux1* mutant's auxin resistant phenotype

[Mahar and Martindale (1980), Pickett *et al.* (1990)]. Therefore, a strategy that would increase the intracellular auxin (root-inducing stimuli) concentration may successfully breach a putative competence threshold and induce adventitious rooting.

1.5.3 Model species

Arabidopsis thaliana

Small physical and genome size [ca. 145 Mbp haploid DNA content; Arumuganathan and Earle (1991)] and short generation time (4-5 weeks) make *A. thaliana* ideal for the study of developmental genetics. Additionally, the multinational *Arabidopsis* Genome Initiative aims to sequence the entire genome of this species, which would facilitate identification of orthologous genes in other species and the isolation of genes from wild-type and mutant plants. The cornerstone of *Arabidopsis* research into plant development depends on the identification of mutations affecting a process or structure, isolation, cloning and sequencing of the gene responsible, determination of expression both spatial and temporal, and the elucidation of the (direct and indirect) interactions of gene product. Mutations may arise naturally or be created, e.g., chemically with ethylmethylsulphonate (EMS), or by transposon or T-DNA (transferred DNA) insertion which induces deactivation of gene function [Sundaresan (1996), Azpiroz-Leehan and Feldmann (1997)]. Such deactivation routinely results in a recessive phenotype. An alternative technique for mutagenesis involves T-DNA activation tagging with transcriptional enhancer sequences (Walden *et al.*, 1994) resulting in dominant overexpressing mutations that allow direct selection of primary transformants. Isolation and characterisation of the mutated gene can be achieved via plasmid rescue, genomic library construction or inverse polymerase chain reaction utilising the known DNA sequence used for insertion mutagenesis as tags [Gibson and Somerville (1993), Azpiroz-Leehan and Feldmann (1997)].

Enormous leaps in our understanding of plant development are being achieved by the investigation of *A. thaliana* as a model species to provide information on plant biology directly transferable to tree species, e.g., the investigation of flower initiation by ectopic expression of the *A. thaliana LEAFY* gene in Aspen (Weigel and Nilsson, 1995) or in the elucidation of lateral root development (Malamy and Benfey, 1997). Particularly relevant to this thesis is the discovery of gene mutations that impact lateral and possibly adventitious root initiation and development [Hobbie and Estelle (1994), Walden and Lubenow (1996), Leyser (1997)]. For example, analysis

of the wild-type and mutated auxin resistant phenotypes of *A. thaliana* have led Bennett *et al.* (1996) to hypothesise that the *AtAUX1* gene is involved in auxin transport across the plant cell membrane.

Merits of a Prunus woody model

Although *A. thaliana* provides an excellent tool for the elucidation of the developmental processes of rooting there are several reasons why a complementary woody model is required. The principals established in any model species should be applicable to other species and therefore require validation. Additionally, there are limitations to the capacities of any one species to adequately model the aspects of all others. For instance, mature perennial woody plants by definition have intrinsic physiological differences to non-woody short-lived *A. thaliana*. Thus, particular problems associated with being a woody plant may often only be adequately reproduced in a woody background (e.g., maturation and rooting recalcitrance). In this study, I have utilised two species of cherry native to the UK, *Prunus avium* (wild cherry) and a fastigiate genotype of *Prunus padus* (bird cherry).

Diploid (2n=2x=16) *Prunus avium* has a relatively small genome compared to other tree species, containing a haploid nuclear DNA content approximately twice that of *A. thaliana* [338 Mbp; Arumaganathan and Earle (1991)]. Potentially, a small genome size would facilitate easier construction and screening of genomic libraries for the identification of orthologous genes identified as affecting adventitious rooting in *A. thaliana*. Many years of cherry fruit tree breeding and a genome-mapping project mean Wild cherry has a well-defined genetic background. A tentative isozyme linkage map for *Prunus* has been developed (Boskovic *et al.*, 1997) which could provide breeders with information on which to base crosses and identify superior progeny. This species is utilised within the forestry and horticulture (ornamental and fruit production) industries. In forestry, it can produce high value hardwood timber over a relatively short crop rotation of 55-70 years (Pryor, 1985). Wild cherry has been included in several genetic improvement programmes throughout Europe [Ducci *et al.* (1991), Nicoll (1993)] and is appropriate for planting on the relatively fertile soils of lowland farm woodlands. Several options are available for the conventional vegetative propagation of elite clonal cherry, including leafless hardwood, leafy summer cuttings (Al Barazi and Schwabe, 1985), root cuttings (Ghani and

Cahalan, 1991) and possibly stoolbeds. However, recalcitrance in rooting capacity severely compromises the effectiveness of these techniques.

Prunus padus is tetraploid (2n=4x=32). From a practical point of view, investigation of the molecular genetics of adventitious root formation requires that procedures for the introduction of transgenes into model plants, and the efficient recovery of putative transformants be attained. The regeneration of transgenic plants is often the weak link in many plant genetic transformation strategies. However, a fastigiate genotype of this species (Hammatt, 1993a) readily regenerates adventitious shoots from leaves, thus rendering it of considerable merit as a candidate woody model since *Prunus avium* remains recalcitrant towards adventitious shoot regeneration. In addition, this species is reported to be rooting recalcitrant (Hammatt, 1993a), which provides an ideal background within which to assess hypotheses aimed at elucidating the molecular genetics of rooting competence and maturation.

1.6 Overview of result chapters

It is clear that a molecular picture of auxin action and root initiation are taking shape. However, the physiological association with these processes and maturation is far from fully understood. Experiments in this thesis are designed to explore these gaps. Results chapters had these primary objectives:

1.6.1 Chapter 3.

The rate at which 'apparent rejuvenation' of mature trees (assessed as increased rooting competence) occurs during micropropagation has been postulated to be a function of subculturing frequency, but previous studies have failed to discern subculture number from total time *in vitro*. For the first time, this was studied in *P. avium* shoot cultures of identical age, which had undergone different numbers of subcultures.

1.6.2 Chapter 4.

Gibberellins (GAs) can influence rooting. Their involvement in, or even the control of, the maturation process has been postulated. E*x vitro* and hedged mature trees were treated with GAs of differing structure-activity and effects on growth and rooting assessed.

1.6.3 Chapter 5.

As a possible target species for transformation with the *AtAUX1* gene, methodology was developed to improve adventitious shoot regeneration from leaf explants of *Prunus avium*. The effects of genotype, wounding, surfactant, and silver nitrate were assessed.

1.6.4 Chapter 6.

The first part of this chapter is concerned with refining the technology for transforming *P. avium*, and the latter, focuses on production of *35S::AtAUX1* transgenic *P. padus*. Problems encountered during transgenesis, and possible solutions, are discussed.

1.6.5 Chapter 7.

The adventitious rooting of putative *35S::AtAUX1 Prunus padus* was investigated, and the possible reasons for the observed rooting phenotype were explored.

2. Origins and Maintenance of *Prunus avium* and *P. padus* Cultures

2.1 Sources of Plant Materials

P. avium

Shoots from mature wild cherry rootstock cvs. F12/1 and Charger (Table *2a*) were excised from scions that had been grafted onto *P. avium x P. pseudocerasasus* cv. Colt rootstock during 1980, and maintained as a field-grown hedge, with hard pruning annually each spring.

British wild cherry accessions (Table *2a*; Figure *2a*) were obtained from woodland locations, as 50-70 mm long stem sections, with 3-5 buds of the previous year's growth, from the crown of the tree. Over several years, during each February, accessions were grafted onto cv. Colt rootstock, and maintained in a peat-based growing medium in an unheated glasshouse. These composite trees provided shoot tips for initiating cultures *in vitro*.

Further details on the sources, dates and methods employed in establishing some of the various *P. avium* shoot cultures described in this thesis can be found in Hammatt and Grant (1993, 1997a).

Table 2a. Origin of the British wild cherry (*Prunus avium*) cultures described in this thesis

Accession	Woodland location of parent tree	UK Ordnance Survey grid reference	Year culture line(s) were initiated
1904	Stoke Row Triangle, Oxfordshire	SU 665 848	1990
1905	" " " "		1994
1906	Chisbury Wood, Wiltshire	SU 269 652	1992
1908	Frydd Wood, Powys	SO 075 901	1992
1909	" " "		1992
1912	Barrowfield Wood, Cumbria	SD 483 912	1992
1919	Lane Wood, Buckinghamshire	SU 989 987	1992
2474	Kilndown, Kent	TQ 697 356	1994
Charger	Cultivar	-	1992
F12/1	Cultivar	-	1994 / 97 / 98 / 99

Fastigiate P. padus

Details on the origin of this fastigiate form of *P. padus* (Figure *2b*) can be found in Hammatt (1993a). Briefly, the author obtained graftwood from a tree located within the University of Nottingham's Botanic Garden, which was subsequently grafted, in February 1990, onto one-year-old *P. padus* seedlings. These composite trees were maintained on a peat-based growing medium in an unheated glasshouse. On the 19th April 1994, shoot tips were excised from these trees to initiate the shoot culture line described in this thesis. Composite trees of both cherry species were located at HRI East Malling, West Malling, Kent.

2.2 Media Preparation and Culturing Conditions

Unless stated otherwise, the following conditions applied. All media were supplemented with 0.6 % (w/v) agar (Merck, Dorset, UK), and adjusted to pH 5.65 prior to autoclaving [5 min., 121 °C; Emperor autoclave (Denley, West Sussex, UK)]. Cultures were maintained, without humidity control, at 24.6 ± 2 °C with a 16 h photoperiod of 60-70 μmol m^{-2} s^{-1} irradiance (PAR) from Phillips 70 W type 84 fluorescent tubes, positioned 25 cm above the shelf on which cultures were located. Irradiance intensities were recorded using a Skye SKP200 meter. Depending on specific experiments, culture media (a complete list of which is described in appendix Table *8b*) were routinely dispensed into a range of containers, the dimensions and construction of which are described in Table *2b*. Where Petri Dishes were used, these were sealed with two or three complete turns of 'Nescofilm' (Nipon Co., Japan).

Table 2b. Dimensions and construction of culture containers

Abbreviation	Name	Dimension(s) or vol.	Construction	Medium vol. (ml)	Supplier
CCPs	Coulter counter pots	30 cm^3	Clear polystyrene body and frosted polyethylene cap	8	Greiner Labortechnik, Glos., UK
PDs	Petri dishes	9 cm dia.	Clear polystyrene	25	ditto
		14 cm dia.		125	ditto
SPDs	Square petri dishes	100 x 100 mm	Clear polystyrene	65	ditto
HJs	Honey jars	350 cm^3	Clear glass body with white polypropylene cap	50	Astell Scientific, UK

2.3 Initiation and Maintenance of Cultures

2.3.1 Establishing shoot cultures

Shoot tips from composite trees of both cherry species were excised, surface-sterilised [10 min. in 10 % (v/v) aqueous commercial bleach solution 'Domestos' (Lever Bros., UK), with not less than 0.5 % (w/v) final NaOCl concentration], and rinsed with sterile water six times. Following rinsing, shoot tips were reduced in length to approximately 5 mm and leaf primordia exceeding 10 mm in length were removed. The resulting explants were transferred singly to semi-solid shoot culture medium in CCPs.

2.3.2 Media and maintenance of cultures

Shoots cultures of *P. avium* (Figure *2c*) were initiated on semi-solid **sh**oot proliferation **m**edium 1 (SHM1) which consisted of growth-regulator-free, modified MS-medium (Murashige and Skoog, 1962; Imperial Laboratories, Dorset, UK) supplemented with 4.4 μM benzyladenine (BA), 0.29 μM gibberellic acid (GA_3), 0.49 μM indole-3-butyric acid (IBA; all growth regulators were obtained from Sigma, Dorset, UK), 1 mM 1,3,5-trihydroxybenzene (phloroglucinol; Fluka, Dorset, UK) and 87.7 mM sucrose (Merck, Dorset, UK), as described previously by Hammatt and Grant (1993, 1997a). After approximately 42 d of culture, and at 28 d intervals thereafter, shoots were transferred to **sh**oot proliferation **m**edium 2 (SHM2) which was of a similar composition but with benzyladenine concentration reduced to 2.2 μM and without GA_3.

P. padus shoot cultures (Figure *2e*) were initiated and maintained on semi-solid **sh**oot proliferation **m**edium 3 (SHM3), which consisted of growth-regulator-free DKW medium (Driver and Kuniyuki, 1984; Imperial Laboratories, Dorset, UK) supplemented with 4.4 μM BA, 49 nM IBA and 166.5 mM fructose (Duchefa, Haarlem, NL). Fructose was used as a carbon source since Hammatt (1993a) observed increased proliferation and reduced hyperhydricity in shoot cultures of this species with fructose compared to those maintained on medium with sucrose.

Unless stated otherwise, cultures from both species, were maintained by separating, with a scalpel, individual shoots from proliferating cultures at approximately 28 d intervals, and transferring these singly to fresh medium.

2.3.3 Basic protocol for rooting cherry shoots

For both species, shoots were excised from donor cultures approximately 28 d after each subculture. Shoots, approximately 10-15 mm in length, were excised below a node, had their basal two-thirds stripped of leaves and were inoculated into rooting medium.

P. avium shoots were inoculated (two per CCP; Figure *2d*) into root initiation and development medium 1 (ROM1) which consisted of growth-regulator-free, semi-solid, modified MS-medium supplemented with 87.7 mM sucrose and 14.7 μM IBA.

Shoots of *P. padus* were inoculated (four per CCP) into semi-solid root initiation medium 2 (ROM2) which consisted of 0.6 % (w/v) agar with or without auxin (various concentrations and auxins used, see individual experiments for details). After 3 d, shoots were transferred (two per CCP) to root development medium 3 (ROM3) for a further 25 d of culture (Figure *2f*). The latter medium consisted of semi-solid growth-regulator-free DKW medium (Driver and Kuniyki, 1984) with 166.5 mM fructose.

Unless stated otherwise, for both species, data on the proportions of shoots that rooted and number of roots per rooted shoot were recorded 28 d after shoots had been transferred to rooting medium.

Figures 2a-f. *P. avium* and *P. padus*: The species of cherry described in this thesis

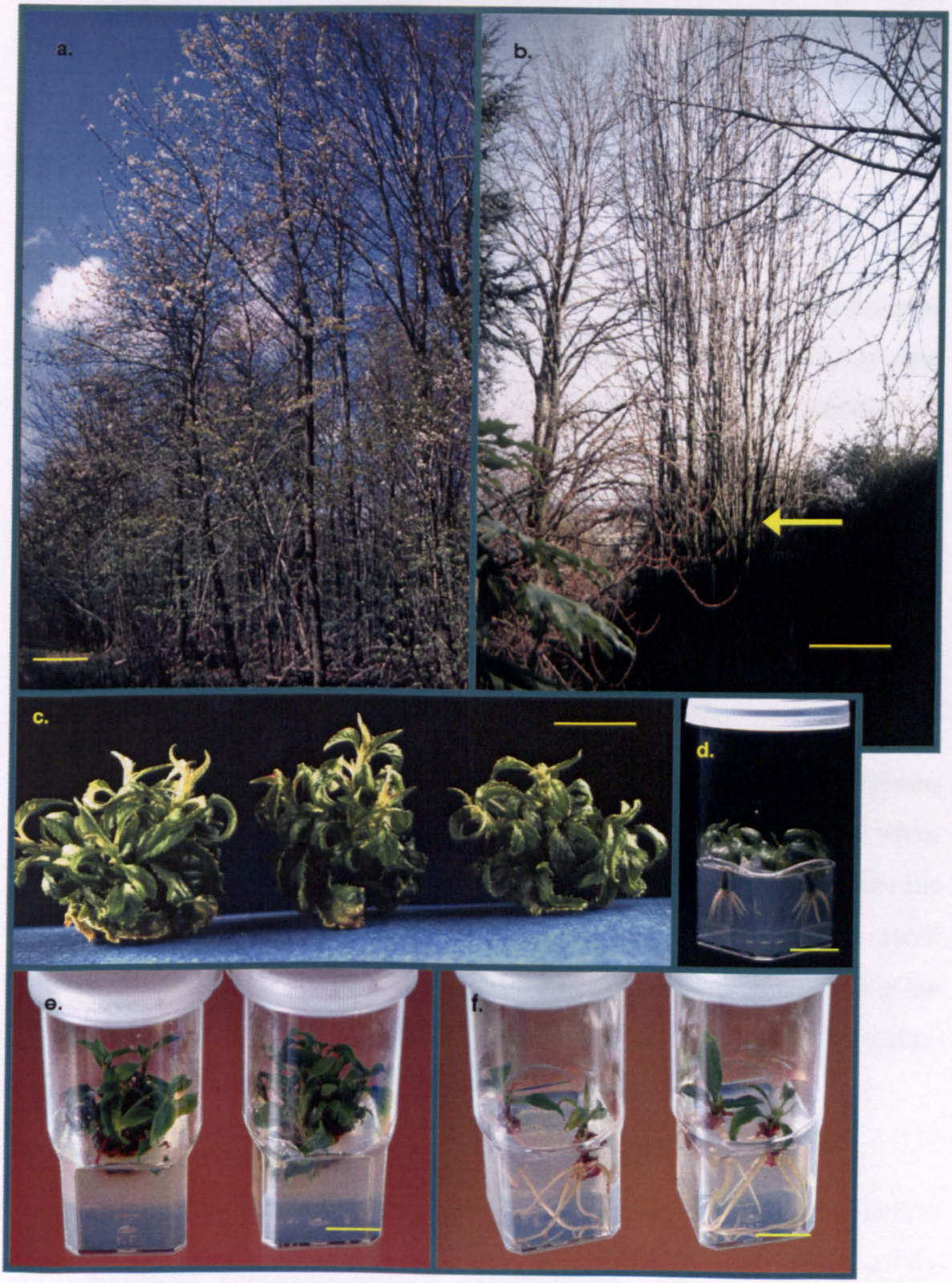


Figure key:

a. Mature *Prunus avium*

b. Fastigiate *Prunus padus* (arrowhead marked)

c. *P. avium* shoot cultures

d. Rooting *P. avium* shoots in a CCP

e. *P. padus* shoot cultures

f. Rooting of *P. padus* shoots

Scale bars for trees = 1 m, and for *in vitro* cultures = 10 mm

3. Factors Influencing the Rate at Which Adventitious Rooting of Mature *Prunus avium* Changes During Long-term Culture

(Results of experiment *3.1* published in: Grant and Hammatt, 1999; a copy of which is appended)

3.1 Introduction

3.1.1 Micropropagation

Micropropagation involves the growth and multiplication of excised plant tissues/organs under aseptic conditions *in vitro* (George, 1993). Cultures are usually maintained in liquid or on agar-solidified medium containing defined essential macro- and micro-nutrients (or complex undefined additions, e.g., coconut milk or casein enzymatic hydrolysate), a carbon source (predominantly sucrose), and are frequently supplemented with growth regulators [Gaspar *et al.* (1996), Kende and Zeevaart (1997)]. Division of cultures and transfer to fresh medium (subculturing) is carried out periodically to prevent the possibility that culture growth would become limited by the depletion of nutrients, overcrowding, the build up of phytotoxic metabolites, and/or to promote the multiplication of new cultures.

Several approaches are available for the propagation of plant tissues *in vitro*, including indirect/direct (i.e., with or without the prior formation of callus) embryogenesis or adventitious shoot regeneration (George, 1993). Alternatively, during the culture of shoot tips or stem node sections axillary shoot proliferation can be stimulated by exogenous cytokinin and/or decapitation, possibly, overcoming auxin-mediated apical dominance. By manipulation of the cytokinin/auxin ratio in favour of auxin, roots rather than shoots can be induced [Skoog and Miller (1957), Murashige and Skoog (1962)], thereby initiating autonomous plants.

3.1.2 The effect of micropropagation on mature tree tissues

For a number of tree species, micropropagation has circumvented some of the consequences of ageing and maturation by improving shoot vigour and adventitious rooting [*Malus pumila*, Webster and Jones (1989), Noiton *et al.* (1992); *Fraxinus excelsior*, Hammatt (1994); and *Prunus avium*, Hammatt and Grant (1993, 1997)]. Furthermore, enhanced rooting potential has been sustained *ex vitro*, in cuttings taken from hedges or stoolbeds (effectively a severely pruned hedge, created by partial mounding of earth over coppiced stockplants to promote etiolated

growth) planted with micropropagated plants [*Prunus insititia*, Howard *et al.* (1989); *Pyrus communis*, Jones and Webster (1989); *Ficus benjamina*, Kristiansen (1991); *Malus pumila*, Jones and Webster (1992); *Prunus avium*, Hammatt (1998)]. For example, hedged, micropropagated plum stockplants still produced cuttings with improved rooting potential nine years after their establishment (Howard *et al.*, 1989), and with cherry, for at least six years (Hammatt, unpublished data). In contrast, only transient improvements in vigour *ex vitro* have been observed in other studies [*Malus pumila*, Jones and Hadlow (1989); Webster and Jones (1992); *Prunus insititia*, Howard *et al.*, (1989)]. With apple, results suggest a positive correlation between the total time that shoot lines have spent in culture and the subsequent ease of conventional vegetative propagation (Webster and Jones, 1992).

Long-term micropropagation of mature trees promotes the gradual appearance of characteristics typical of the juvenile development phase. It is implausible that this phenomenon results from the gradual elimination of viruses and other pathogens or the selection of genetic variants, as these improvements have occurred in apparently pathogen-free cultures, and results were replicated and reproducible, in shoot cultures with no observable aberrant phenotype [Hammatt and Grant (1993), Grant and Hammatt (1999)]. Hence, this process has been hypothesised to result from physiological rejuvenation [Webster and Jones (1989), Jones and Webster (1992), Noiton *et al.* (1992), Hammatt and Grant (1993, 1997)] and termed 'apparent rejuvenation' by a number of these authors. In addition to the evidence cited above, further support for this view is demonstrated by the transient inhibition of flowering *ex vitro* in a number of species [*Malus pumila*, Jones and Hadlow (1989); *Eucalyptus grandis x urophylla*, Yang *et al.* (1995); *Prunus avium*, Hammatt (1999)], which suggests that some degree of developmental phase reversal may occur during micropropagation. However, relatively rapid changes in putative molecular markers for developmental phase, e.g., the concentration of a protein, that differs in relative abundance between developmental phases (Hand *et al.*, unpublished data), occured *in vitro* prior to any significant improvement in adventitious rooting.

Little is known about the underlying mechanism(s) evoking the process of 'apparent rejuvenation'. The gradual changes that occur during micropropagation, and continue *ex vitro*, cannot be attributed directly to an abrupt loss of florogenic ability. Models of maturation and chronological ageing were discussed previously (Section *1.3*). One such model proposed by

Moorby and Wareing (1962) postulates that physiological ageing results from reduced apical dominance and nutritional deprivation in chronologically older stems, upon which, longer-term phenomena such as induction of sexual competence (maturation or ontogenetic ageing) act. During micropropagation, nutrient and carbohydrate deprivation are abated. In apple, Webster and Jones (1989), and Noiton *et al.* (1992) concluded that the rate at which 'apparent rejuvenation' occurred was a result of subculturing frequency. However, both studies failed to distinguish between subculture number and total time in culture during their experimentation.

3.1.3 The need for developmental phase markers

The ability to monitor the development phase of plant tissues, via molecular or growth regulator markers, may elucidate whether treatments, inducing juvenile-like traits, act upon these directly or indirectly, identifying the most effective. Additionally, markers would aid the location, within donor plants, of the most receptive (i.e., juvenile) plant tissue from which to propagate. Markers may be expressed in a phase-specific manner or differ relatively in abundance between phases. A number of such putative markers have been identified:

Protein markers: A 12 kDa protein, which is relatively more abundant in juvenile-phase shoot tips of *Prunus avium* has been identified (Besford, *et al.*, 1996). Similarly, Bon (1988) isolated, from *Sequoiadendron giganteum*, a 16 kDa juvenile phase-specific protein which could not be detected in shoots of mature origin but reappeared in serially grafted, 'apparently rejuvenated' shoots. Phase-related proteins have also been identified in *Sequoia sempervirens* (Bon *et al.*, 1994) and *Castanea sativa* (Amo-Marco *et al.*, 1993). In our laboratory, a 23 kDa protein in the oxygen-evolving complex of photosystem II was identified in *P. avium*, *Fraxinus excelsior* and *Quercus robur* as a marker by virtue of being expressed in greater abundance in the juvenile than the mature phase (Hand *et al.*, unpublished data).

Endogenous IAA and ABA concentration: Several studies suggest that the endogenous concentration and/or ratio of indole-3-acetic acid (IAA) and abscisic acid (ABA) may be correlated with development phase, and hence may be, a potential marker. For example, Fouret *et al.* (1986) reported a correlation between the IAA:ABA ratios of *Sequoia sempervirens* shoot cultures and the age of stock plants from which they originated. Similarly, Oliveira and Browning (1993) observed that IAA:ABA ratios of callus, cultures derived from mature *Prunus avium*, were

lower (0.18-0.2) than those derived from juvenile counterparts (1.64-1.76). Interestingly, Noiton *et al.* (1992) found that, during prolonged micropropagation of apple shoot cultures, the endogenous ABA concentration decreased, increasing the IAA:ABA ratio, which also correlated with improved rooting capacity.

3.1.4 Chapter aims

Experiments were designed to test the hypothesis that the rate at which adventitious rooting capacity improved during micropropagation was a function of total time in culture, rather than subculture frequency, by varying the subculture interval of cultures of the same age. Frequently, light fluence *in vitro* is modified to that *in vivo*. The effect of extending subculture interval from 28- to 42 and 84 d on rooting capacity was determined under equalised irradiance intensity. However, to achieve subculture intervals beyond 42 d, *P. avium* cultures needed to be incubated at low temperatures. Changes in IAA and ABA concentration and ratio were recorded during the early stages of culture initiation, a period where Hand *et al.* (unpublished data) found a dramatic increase in their 23 kDa protein.

3.2 Materials and Methods

Unless otherwise stated, shoot cultures of mature *Prunus avium* c.v. F12/1 were initiated, maintained, excised and transferred to rooting medium as described previously (Chapter *2*).

3.2.1 Experiment 3.1: Subculture intervals of 28 and 42 d

Shoot cultures were initiated on the 18th June 1996 on SHM1, transferred after 42 d of culture to SHM2, and thereafter maintained by subculturing at 28 d intervals.

After 105 d of culture, three shoot culture lines were selected arbitrarily and subdivided into two sub-lines, one of which continued to be subcultured every 28 d, and the second every 42 d. When sufficient shoots became available to assess rooting, 28 d after each subculture, up to 20 shoots per sub-line were excised and transferred to ROM1.

To extend the subculture interval beyond 42 d, cultures were transferred after 133 d of culture to a cooled incubator [4 ± 2 °C, 16 h photoperiod of 10-20 μmol m^{-2} s^{-1} irradiance (PAR) from 8 W type 33 fluorescent tubes]. These were subcultured in May and November 1997. At the latter subculture (509 d of culture), three culture lines were selected arbitrarily, returned to 24.6 ± 2 °C, and subcultured at 28 d intervals. At the two subsequent subcultures, 20 shoots were excised from each sub-line and transferred to ROM1.

3.2.2 Experiment 3.2: Extending subculture interval at 4 ± 2 °C

Shoot cultures were initiated on the 29th July 1998 on SHM1, transferred after 42 d of culture to SHM2, and thereafter maintained by subculturing at 28 d intervals.

After 161 d of culture, three shoot culture lines were selected arbitrarily. Each culture line was subdivided into two sub-lines, one of which continued to be subcultured every 28 d and maintained at 24.6 ± 2 °C, and the second was transferred to a cooled incubator [4 ± 2 °C, 16 h photoperiod of 10-20 μmol m^{-2} s^{-1} irradiance (PAR) from 8 W type 33 fluorescent tubes] and subcultured at 84 d intervals. To equalise the irradiance intensity for cultures with different subculture intervals, those maintained at 24.6 ± 2 °C were enclosed within a perspex cloche covered with neutral density film (Lee Filters, Hampshire, UK) which reduced irradiance intensity to 15 μmol m^{-2} s^{-1} (PAR).

After each subculturing, 12 arbitrarily selected cultures from each sub-line which had been maintained at 4 °C were returned to 24.6 ± 2 °C and subcultured. After 28 d of culture, 20 shoots were excised from each sub-line and transferred to ROM1.

3.2.3 Experiment 3.3: Maintaining cultures at three irradiance intensities

Shoot cultures were initiated in two replicate experiments on 29th July and 15th September 1998 on SHM1, transferred after 42 d of culture to SHM2, and thereafter maintained by subculturing at 28 d intervals.

After 126 d of culture, two shoot lines were selected arbitrarily and both were subdivided into three sub-lines. Sub-lines were maintained under either 12, 25 or 50 μmol m^{-2} s^{-1} irradiance (PAR) intensities (70 W type 33 fluorescent tubes with a 16 h photoperiod) by their enclosure within perspex cloches covered with neutral density film. When sufficient shoots became available, they were transferred to ROM1 at each subculture.

3.2.4 Experiment 3.4: Determining rooting during the early stages of culture establishment

Shoot cultures were initiated on the 16th June 1999 on SHM1, transferred after 42 d of culture to SHM2, and thereafter maintained by subculturing at 28 d intervals.

Except for three shoots that were selected arbitrarily after 42 d of culture to propagate clonal shoot culture lines, cultures were propagated by subculturing only the axial shoot (i.e., no axillary shoots were subcultured). Directly after surface-sterilising shoot tips, at 42 d of culture and thereafter at 28 d intervals to 182 d of culture, 20 cultures were selected arbitrarily and their axial shoots were excised and transferred to ROM2. After 238, 294 and 350 d of culture, up to 20 axial shoots from each of the three clonal shoot lines were transferred to ROM2.

3.2.5 Experiment 3.5: Determining endogenous IAA and ABA concentrations in shoot cultures from their initiation

Initiating shoot cultures and sampling

Shoot cultures were initiated on the 29th July 1998 on SHM1, transferred after 42 d of culture to SHM2, and thereafter maintained by subculturing ever y 28 d.

Shoot tips were sampled for determination of IAA and ABA content. Three samples of 10 shoot tips (2-3 cm in length) were taken directly from the tree, one from each of ten trees, and

flash frozen in liquid nitrogen. Further samples (3 x 10 shoot tips) were selected arbitrarily from shoot cultures after 7, 21, 42 d of culture, which resulted in the destruction of that particular culture line. At 115 and 148 d of culture samples were taken from four independent clonal shoot culture lines in proportion to the number of available cultures. Thus, samples at 115 d and 148 d consisted of 6, 7, 8, 8 (total=29) and 14, 12, 12, 6 (total=44) shoots from these four lines. In identical proportions, shoots were excised from these cultures and transferred to ROM1. Shoots were partitioned into stem, leaf and callus tissues from 42 d and analysed separately.

Determination of IAA:ABA ratio

IAA and ABA were extracted by homogenising each tissue sample in 20 ml of cold 80% (v/v) methanol containing 20 mg l^{-1} butylated hydroxytoluene, 200 ng l^{-1} of indole-2,4,5,6,7-d5-3 acetic acid standard ([$^2H_5$]-IAA)(MSD isotopes Merck Frosst Canada Inc.) and 200 ng l^{-1} of hexadeutero-(+-) cis, trans abscisic acid standard ([$^2H_6$]-ABA) (Rivier *et al*, 1977) and extracted by stirring overnight at 4 °C. After filtration the extracts were purified using the method of Kamboj *et al* (1999).

Analysis by gas chromatography mass spectrometry (GC-MS)

Derivatised samples were analysed using a VG TRIO_1 GC-MS system. The BP1 (S. G. E. Plc) capillary column (25 mm x 0.25 mm i.d. x 0.25 d.f.) was coupled directly to the ion source with an interface temperature of 275 °C and Helium carrier gas inlet pressure at 40 k Pa. The MS source temperature was 200 °C and electron energy 70 eV. The injector was used in the splitless mode at a temperature of 250 °C. After injection of ABA or IAA samples (1 µl) the GC oven was maintained at 60 °C for 0.7 min. with the splitter closed, after which time the splitter (50:1) was opened, and 0.3 min. later the oven temperature increased at 25 °C $min.^{-1}$ to 215 °C and then at 5 °C $min.^{-1}$ to 250 °C for ABA, or at 27 °C $min.^{-1}$ to 200 °C and then at 5 °C $min.^{-1}$ to 235 °C for IAA. For quantification by Selected ion Monitoring (SIM) the VG Lab-Base data system software was used to monitor responses to ions of m/e 162 and 190 for Me-ABA and 166 and 194 for Me-[$^2H_6$]-ABA, and 202 and 261 for Me-TMS-IAA and 207 and 266 for Me-TMS-[$^2H_5$]-IAA. Responses were integrated and the amounts of endogenous ABA and IAA computed using the VG-Lab-Base software from the ratios 190/194 and 261/266 respectively, using calibrations relating these ratios to the appropriate molar ratios.

3.2.6 Experimental designs

Unless otherwise stated, all experiments were randomised in blocks, containing one CCP per culture/treatment, and one shoot per CCP.

3.2.7 Statistical analyses

Statistical analyses were carried out with Genstat V software (Genstat 5 Committee, 1993). Generalised linear models (McCullagh and Nelder, 1989), with binomial error and the logit link function, were used to compare the proportions of shoots that rooted. To compare the number of roots per rooted shoot, generalised linear models with positive poisson error and a link function of log (mean -1) were used (Ridout and Demetrios, 1992). The threshold for statistical significance was taken to be the P=0.05 probability level.

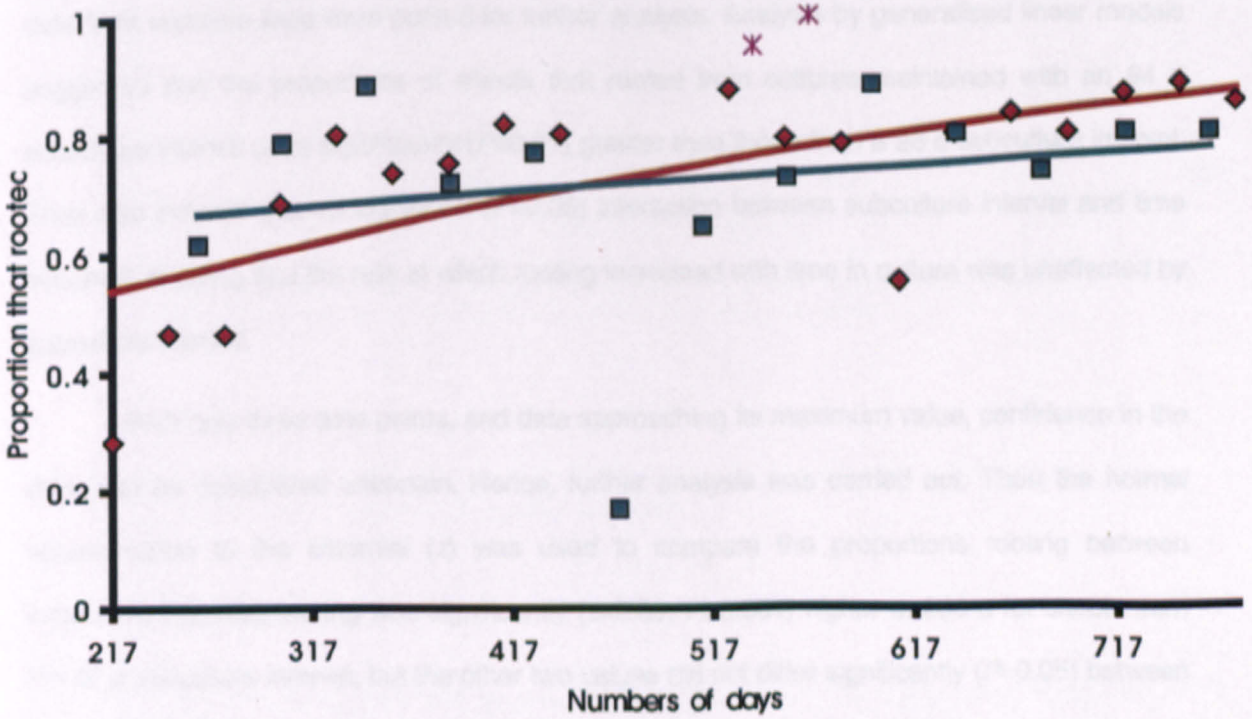
3.3 Results

3.3.1 Experiment 3.1: Effects on rooting of 28 and 42 d subculture intervals

The proportion of shoots that rooted

Overall, the proportions of shoots that produced adventitious roots increased with time in culture ($P<0.001$; Figure *3a*). Overall, rooting from each line differed ($P<0.001$), but there were no significant ($P=0.05$) interactions between culture line and subculture interval. Hence, data from replicate lines were pooled for further analysis. A significant interaction ($P<0.001$) between subculture interval and time in culture suggests that the rate at which rooting increased differed between shoots subcultured at 28 and 42 d intervals, although, time in culture had the greater significance to the improvement in rooting.

Figure 3a. Proportions of *P. avium* cv. F12/1 shoots that produced adventitious roots during micropropagation. Shoots were excised from cultures maintained under 60 µmol m^{-2} s^{-1} irradiance (PAR) with 28- (diamonds; predicted values = ——) and 42 d (squares; predicted values = ——) subculture intervals at 24 ± 2 °C, or under 10-20 µmol m^{-2} s^{-1} irradiance and an extended subculture interval at 4 ± 2 °C (crosses)



Values were derived from 20 shoots per treatment replicated with 3 culture lines
Data were recorded 28 d after shoots were transferred to rooting medium

Data suggest the proportions of shoots that rooted from cultures that had been maintained at 4 ± 2 °C, with an extend subculture interval, rooted at similar proportions to shoots from cultures maintained with 28 or 42 d subculture intervals (Figure *3a*).

Number of roots per rooted shoot

Overall, root number (data not shown) increased with time in culture ($P<0.05$) and differed between shoot lines ($P<0.001$) and subculturing intervals ($P<0.001$). However, there was no significant ($P=0.05$) interaction between subculture interval and time in culture, suggesting that the rate at which root number increased with time in culture was unaffected by subculture interval.

3.3.2 Experiment 3.2: Effect on rooting of extending subculture interval, by maintaining shoot cultures at 4 °C

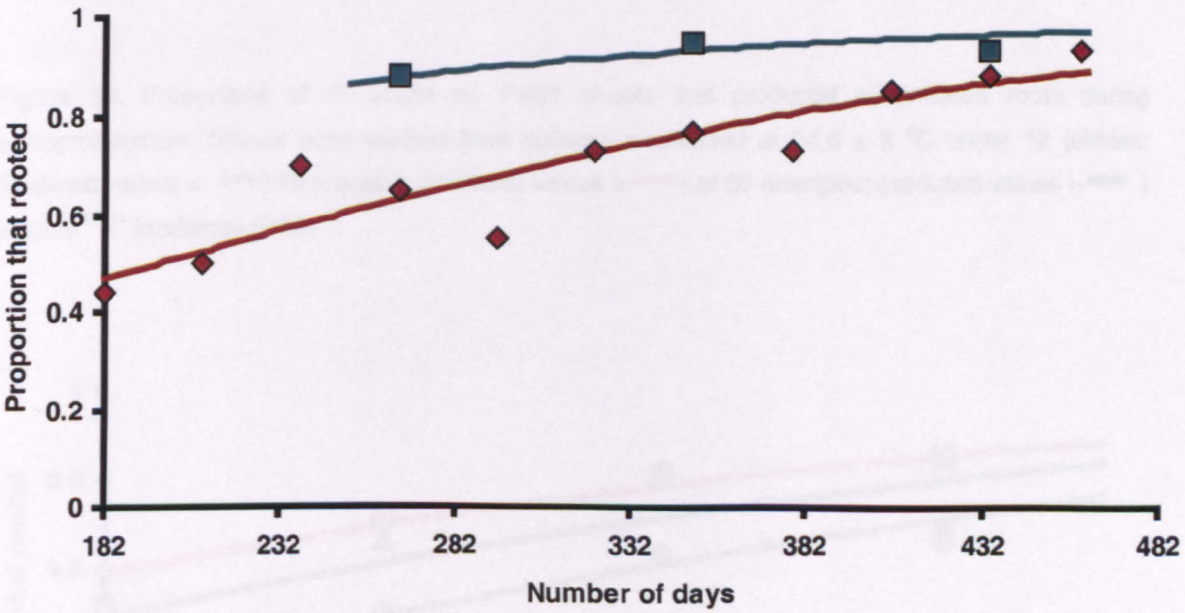
Overall, the proportions of shoots that rooted increased with time in culture ($P<0.001$; Figure *3b*). There were no significant ($P=0.05$) interactions between culture line and subculture interval, so data from replicate lines were pooled for further analysis. Analysis by generalised linear models suggested that the proportions of shoots that rooted from cultures maintained with an 84 d subculture interval were significantly ($P<0.01$) greater than those from a 28 d subculture interval. They also indicate that no significant ($P=0.05$) interaction between subculture interval and time occurred, implying that the rate at which rooting increased with time in culture was unaffected by subculture interval.

With only three time points, and data approaching its maximum value, confidence in the data may be considered uncertain. Hence, further analysis was carried out. Then the normal approximation to the binomial (z) was used to compare the proportions rooting between subculture intervals, rooting was significantly ($z=2.89$, $P=0.004$) higher at 266 d for shoots from the 82 d subculture interval, but the other two values did not differ significantly ($P=0.05$) between subculture intervals.

Number of roots per rooted shoot

Overall, root number increased with time in culture ($P<0.05$; data not shown). There were no significant ($P=0.05$) differences among shoot lines, nor significant interactions between shoot line and subculturing interval, or subculture interval and time in culture. This suggests that the increase in root number with time in culture was unaffected by subculture interval.

Figure 3b. Proportions of *P. avium* cv. F12/1 shoots that produced adventitious roots during micropropagation. Shoots were excised from cultures maintained under 15 µmol m^{-2} s^{-1} irradiance (PAR) with either 28- (diamonds; predicted values =) or 84 d (squares; predicted values =) subculture intervals at 24- and 4 ^{0}C, respectively



Values were derived from two experiments of 20 shoots per treatment replicated with 3 culture lines
Data were recorded 28 d after shoots were transferred to rooting medium

3.3.3 *Experiment 3.3: Effect of maintaining shoot cultures at different irradiance intensities on adventitious rooting capacity*

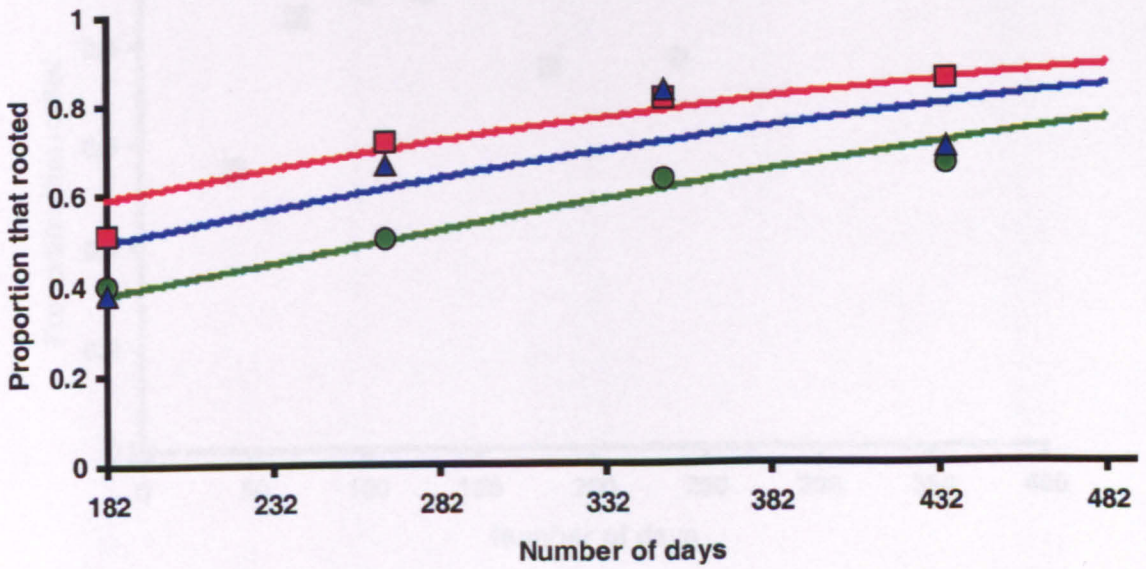
The proportion of shoots that rooted

Overall, rooting increased with time in culture ($P<0.001$; Figure *3c*). There was no significant ($P=0.05$) interaction between culture line and irradiance intensity, so data from replicate lines

were pooled for further analysis. Overall, there were significant differences in the proportions of shoots that rooted from cultures maintained under different irradiance intensities ($P<0.001$). Shoot cultures maintained under 12 μmol m^{-2} s^{-1} irradiance had lower proportions of shoots that rooted than where cultures had been maintained under 25 ($P<0.001$) or 50 ($P<0.05$) μmol m^{-2} s^{-1} irradiance. The proportions of shoots that rooted were just significantly ($P<0.05$) different between 25 and 50 μmol m^{-2} s^{-1}. However, there were no significant ($P=0.05$) differences among the proportions of shoots that rooted, or interactions between irradiance and time in culture suggesting the rate at which the proportions of shoots that rooted increased with time in culture was not influenced by irradiance intensity.

There were no consistent effects on the number of roots per rooted shoot with time in culture (data not shown).

Figure 3c. Proportions of *P. avium* cv. F12/1 shoots that produced adventitious roots during micropropagation. Shoots were excised from cultures maintained at 24.6 ± 2 °C under 12 (circles; predicted values =) 25 (squares; predicted values =) or 50 (triangles; predicted values =) μmol m^{-2} s^{-1} irradiance (PAR)



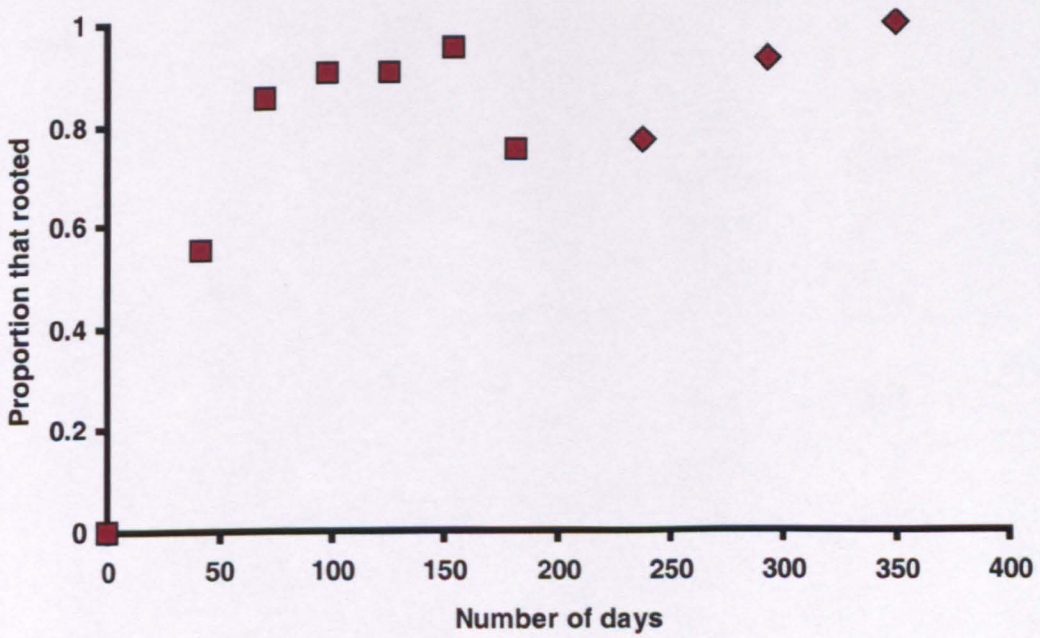
Values were derived from two experiments of 20 shoots per treatment replicated with 3 culture lines
Data were recorded 28 d after shoots were transferred to rooting medium

3.3.4 *Experiment 3.4: Rooting during the initial phase of culture establishment and apparent rejuvenation*

The proportion of shoots that rooted

Overall, the proportions of shoots that rooted increased with time in culture ($P<0.001$; Figure *3d*). Shoots which were transferred to ROM2 immediately after undergoing surface sterilisation (0 d) failed to root. Rooting capacity increased rapidly to asymptote between 70-154 d. Analysis of the data suggests there was a significant decline ($P<0.01$) in the proportion of shoots that rooted in the final sample taken after 182 d of culture from the initial population of non-clonal cultures. However, the proportion of shoots, excised from three clonal shoot lines initiated from the original population, that formed roots, increased with time in culture ($P<0.001$).

Figure 3d. Proportions of *P. avium* cv. F12/1 shoots that produced adventitious roots during micropropagation. Shoots were excised from the initial population of cultures (squares) and then from clonal shoot lines produced from this population (diamonds). Cultures were maintained under 60 µmol m^{-2} s^{-1} irradiance (PAR) at 24.6 ± 2 °C with a 28- subculture interval and rooted on ROM2



Values were derived from 20 shoots per sample, and replicated with 3 clonal lines from 328 d
Data were recorded 28 d after shoots were transferred to rooting medium

3.3.5 *Experiment 3.5: The IAA and ABA concentration during the initial phase of culture establishment and apparent rejuvenation*

There was a significant rapid decline in the endogenous concentration of ABA between samples taken at 0 and 7 d of culture (Table *3a*), resulting in an increased IAA:ABA ratio. Thereafter, no significant trend in the concentrations of IAA, ABA or the ratio of the two with time in culture could be detected in whole cultures, shoots plus leaves or basal callus.

Table 3a. The concentrations of IAA and ABA in mature *P. avium* cv. F12/1 shoots sampled directly from the mother tree and during micropropagation, and the proportions of shoots that rooted on ROM2

	Concentration (ng g^{-1} fresh tissue)					
	Number of days since cultures initiated					
	0	7	21	42	115	148
Whole cultures						
IAA	17.1	8.4	4.6	36	58.6	28.6
ABA	480.6	10.4	13.6	45.9	196.1	67.3
IAA:ABA	0.036	0.81	0.34	0.79	0.30	0.43
Shoots +leaves [a]						
IAA	17.1	8.4	4.6	8.4	45.6	16.2
ABA	480.6	10.4	13.6	16.6	183.9	48.9
IAA:ABA	0.036	0.81	0.34	0.51	0.25	0.33
Basal callus						
IAA	*	*	*	27.6	13.0	12.4
ABA	*	*	*	29.3	12.2	18.4
IAA:ABA	*	*	*	0.91	1.07	0.67
Rooting (%)	*	*	*	*	52	64

[a] Significant quantities of basal callus were not present on shoots until the sample taken at 42 d of culture; therefore, values from 0 – 21 d of culture for whole cultures are approximately = shoot + leaves, and are presented in grey for this reason

Rooting data were collected 28 d after shoots were transferred to rooting medium

*No data available

3.4 Discussion

3.4.1 Subculture interval vs. time in culture

For the first time, shoot cultures of *P. avium* were produced of the same age, but as time progressed they differed in the number of subcultures they had received. These provided the means to test the hypothesis that the rate at which adventitious rooting improved during micropropagation was either a function of time in culture or number of subcultures.

Results from this study were consistent with previous reports in which adventitious rooting improved during prolonged micropropagation [Webster and Jones (1989), Noiton *et al.* (1992), Hammatt and Grant (1993, 1997)]. However, the present study suggests that time spent in culture is the predominant factor leading to improved rooting, and not subculture frequency *per se*, as had been suggested (but not proven experimentally) previously [Webster and Jones (1989), Noiton *et al.* (1992)].

Over all experiments, fluence period remained constant. Shoot cultures with subculture intervals of 28 and 42 d were maintained in a growth room at 24.6 ± 2 °C and 60 μmol m^{-2} s^{-1} irradiance (PAR). For *P. avium* shoot cultures, extending the subculture interval beyond 42 d was dependent on culture at low temperature. Hence, these cultures were maintained in a cooled (4 ± 2 °C) incubator and 10-20 μmol m^{-2} s^{-1} irradiance.

In experiment *3.1*, subculture interval (28 vs. 42 d) affected the rates at which the proportions of shoots that rooted increased with time; however, the effect of subculture frequency on rooting was not as great as overall time spent in culture. Furthermore, the proportions of shoots that rooted from incubator maintained cultures (at 4 °C), were greater than those for cultures maintained in the growth room (at 24 °C) with more frequent subcultures. Overall, this suggests that time in culture, rather than subculture interval, had the greater influence on the rate at which rooting improved. Practically, this result suggests that cultures could be kept at 4 °C while rooting improved. However, temperature and fluence differed between the growth room and incubator maintained cultures.

In an attempt to account for the observation that rooting improved in cultures kept at 4 °C, in experiment *3.2*, fluence was kept consistent between growth room and incubator by

reducing the growth room fluence to 15 μmol m^{-2} s^{-1} irradiance. If fluence, rather than temperature difference, were the causative factor increasing the proportion of shoots that rooted from incubator-maintained cultures during experiment *3.1*, removing this difference should have increased the proportions of shoots that rooted from cultures with a 28 d subculture interval to values comparable to the rooting of cultures with a 84 d subculture interval. However, this did not happen: as in experiment *3.1*, still greater proportions of shoots rooted from incubator than growth room maintained cultures.

In a further experiment (*3.3*), temperature and subculture interval were kept constant and fluence was altered. If fluence in the incubator (i.e., 10-20 μmol m^{-2} s^{-1} irradiance) was promotory to the acquisition of increasing rooting competence, then cultures maintained under 12 μmol m^{-2} s^{-1} irradiance (but at 24 °C) might have been expected to have a better rooting performance than cultures maintained under 50 μmol m^{-2} s^{-1} irradiance (approximately growth room irradiance). However, the hypothesis was rejected because cultures under 12 μmol m^{-2} s^{-1} irradiance gave lower proportions of rooted shoots than those at 25 and 50 μmol m^{-2} s^{-1} irradiance.

In all three experiments, the number of roots per rooted shoot, increased with time in culture but was not greatly influenced by subculture interval. Overall, the data suggest that subculture interval and fluence were not factors determining the rate at which rooting improved with time in culture at 4 °C, implying that temperature in the 84 d material, or an unidentified factor (at 24 °C) was involved. A new experiment in which cultures are maintained at 4 °C under various irradiance intensities is recommended. The hypothesis being that if temperature, and subculture interval, are constant there should be no significant difference in rooting performance between the different irradiance intensities.

Maintaining shoot cultures with illumination at 1-4 °C has previously been used to reduce the need for subculturing woody species [Landergan and Janick (1979), Chun and Hall (1986)]. However, the effect of this treatment on the subsequent capacity of cultures to form shoots and roots was not reported. Since seasonal temperature fluctuations have a significant influence on cherry growth *in situ*, cold treatment may have been having an effect on cultures independently of that derived from subculture interval.

Interestingly, data from experiments *3.2* and *3.3*, suggest that rapid, sustainable, differences in competence were established early in the life of each culture line. Similar observations have been made in apple (Grant and Hammatt, 1999). Speculative models for maturation based on cellular switches have been proposed [Hackett *et al.* (1992), Greenwood (1987), Section *1.3.3*]. Should they exist, subculture interval may influence a subset of switches, causing rapid partial resetting of rooting competence; the progressive increase in rooting with time could be mediated by a further cascade of switches, which instigate physiological and biochemical change.

3.4.2 Rooting capacity, IAA and ABA content during the early stages of culture establishment and rejuvenation

Barely any knowledge exists on the rooting of shoots in the period that spans the introduction of a shoot *in vitro* to production of a proliferating clonal shoot line. Principally, this results from the desire to propagate sufficient shoots for the continuance of a clonal line before a statistically valid number of shoots can be routinely dedicated to rooting tests [Webster and Jones (1989), Jones and Webster (1992), Hammatt and Grant (1993, 1997)].

The early establishment period of shoot cultures is interesting for a number of reasons. Data from cherry (this study) and apple (Grant and Hammatt, 1999), suggest that sustainable differences in rooting competencies can be set early in the life of cultures. During the micropropagation of *P. avium*, abundance of a 23 kDa protein, identified as a putative marker of juvenility, increased rapidly between 0 and 42 d of culture (Hand *et al.*, unpublished data).

Shoots were not competent to root at 0 d. However, the proportions of non-clonal and clonal shoots that rooted, increased progressively with time in culture. Non-clonal shoots showed a rapid increase in capacity to root to asymptote between 70-154 d. These shoots were maintained by subculture of the axial shoot only, with axial shoots of the resulting cultures being transferred to rooting medium. However, axillary shoots were used to propagate clonal shoot lines for subsequent rooting. Interestingly, extrapolated, data suggests rooting between these two populations may differ. Possibly, the rate at which enhanced rooting competence is acquired may differ between axial and axillary shoots.

These preliminary data provide evidence that *P. avium* cv. F!2/1 cultures *in vitro* can rapidly acquire the capacity to form roots, in a period of culture previous studies have not studied.

Thus, rooting performance may also be increasing rapidly in a comparable time frame to that during which rapid changes occur in the relative abundance of a 23 kDa protein, postulated to be putative marker of juvenility in some deciduous trees (Hand *et al.*, unpublished data), and in the ABA content of cherry shoots (discussed below).

From a practical point of view, it is pertinent to ask how short can we make the period cultures need to spend *in vitro* and still be able to achieve improved rooting *ex vitro.* As little is known about the relationship between rooting performance *in vitro* and *ex vitro*, it would be valuable to determine if this rapid increase in rooting could be transferred *ex vitro* as has been demonstrated for long-term micropropagated cultures (Section *3.1.2*).

IAA and ABA content

Previously with *P. avium*, endogenous ABA concentration was found to decline progressively with time in culture, and rooting has been correlated to whole culture IAA:ABA ratio (Hammatt and Grant, unpublished data). That this ratio is a marker of rejuvenation was suggested by Oliveira and Browning (1993). However the IAA:ABA ratios observed by Hammatt *et al.* (unpublished data) were greater than those observed by Oliveira and Browning (1993). However, in the former study 200 d of culture had elapsed before the assessment of IAA and ABA concentration commenced, whereas, the latter samples were collected soon after callus cultures were initiated. Thus, from *P. avium* shoots, no data were available on endogenous concentrations of IAA and ABA prior to 200 d of culture.

Data from this study indicate a significant decline in ABA, resulting in an increased IAA:ABA ratio during the first 7 days in culture. Since shoot tips were sampled directly from the tree at 0 d, the initial high ABA concentration cannot be an artefact associated with the initial stress imposed on the explant tissues at establishment. Thereafter, no significant trends could be detected in whole or partitioned samples. Similarly, Noiton *et al.* (1992), observed in apple that the greatest increase in IAA:ABA ratio occurred between subcultures 0 and 4, and thereafter the ratio remained at similar levels. Over the 148 d of culture studied, the ratio of IAA:ABA fluctuated but were intermediate between those observed by Oliveira and Browning (1993) for callus cultures derived from mature and juvenile *Prunus*. This, may indicate that the cherry cultures

were intermediate between mature and juvenile development phases (i.e., a phase of partial rejuvenation). However, to increase competence in the data, further experimentation is required.

4. Application of Gibberellins to *Ex Vitro* and Mature *Prunus avium* stockplants: Effects on Growth and Rooting of Cuttings

4.1 Introduction

4.1.1 Gibberellins

In a continuing quest, 121 endogenous gibberellins (GAs) have so far been identified in higher plants or fungi [Hedden (1999); for a historical review, see, Phinney,(1983)]; however, many of these are physiologically inactive biosynthetic precursors, intermediates or deactivated metabolites (MacMillan, 1997). So far, evidence suggests that in any particular plant, only a subset of these are present, the composition of which may vary with development phase. For instance, of the known GAs, eleven plus 16α, 17-dihydrodihydroxy GA_{25} have been detected in cherry (*P. avium* cv. Stella) so far: five in mature seed, ten in germinating seed, eleven in ten week-old seedlings and seven in mature flowering plants seed [Blake *et al.* (1993), Blake and Browning (1994), Blake *et al.* (2000)].

Gibberellins are diterpenes synthesised from acetate groups of acetyl coenzyme A within the mevalonic acid pathway, which branches from the general terpene biosynthetic pathway at geranylgeranyl diphosphate (MacMillan, 1997). GAs are hydrophobic weak acids with a common C7 (carbon 7) carboxyl group. They are all oxidised variants of the 20 carbon *ent*-gibberellane skeleton (Figure *4a*), either with a four or five-ring structure, where the C19 methyl group of the four ring *ent*-gibberellane skeleton is oxidised to form the fifth (lactone) ring. Structural features of the A/B rings are important in determining high biological activity (MacMillan, 1997). Bioactivity is associated with the presence of a 3β-hydroxyl group, while 2β-hydroxylated GAs are usually inactive [Ingram *et al.* (1984), Spray *et al.* (1984)].

4.1.2 Gibberellin-induced juvenile-like traits

GAs influence plant development in numerous ways (Hooley, 1994). Interestingly, exogenously applied GAs have been found to promote attributes of the juvenile phase, e.g., growth vigour, capacity to form adventitious roots and/or inhibit those of the mature phase (e.g., flowering). *In planta* the effects of GAs may be mediated either by changes in cellular competence to perceive

and/or respond to the stimuli and/or alteration of the endogenous concentration(s) and/or composition of bioactive GAs.

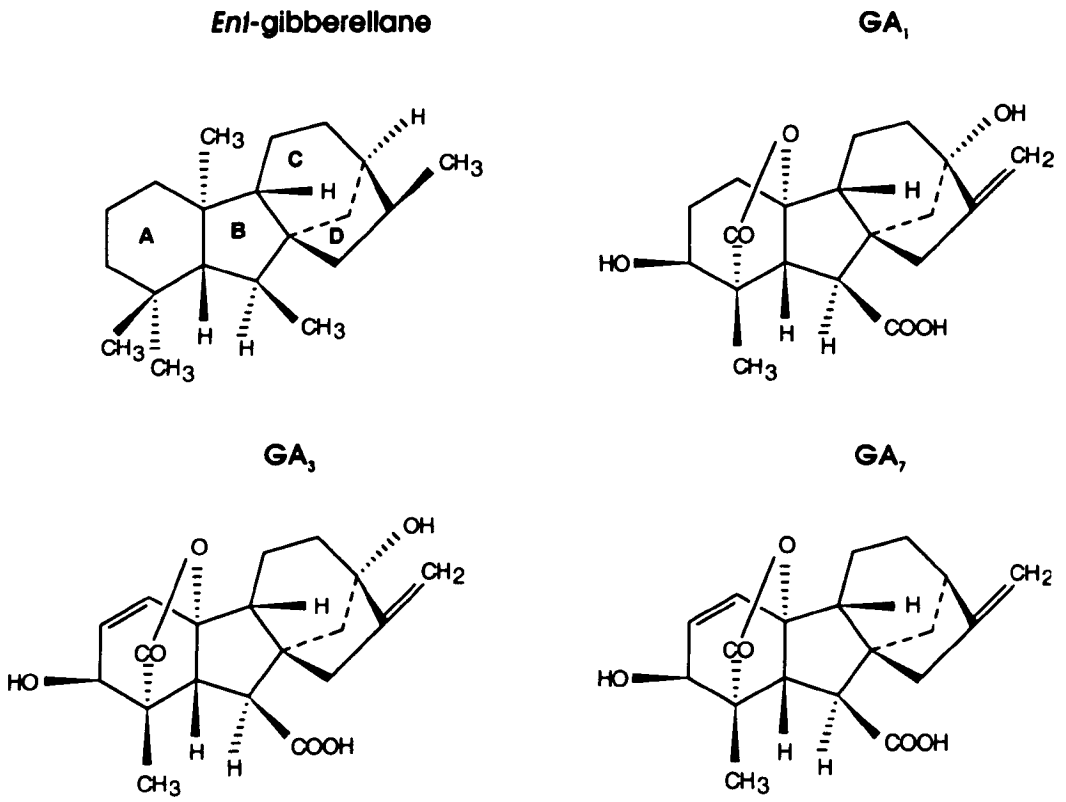
In *P. avium*, the 12α, 13-hydroxy GAs, GA_{32}, GA_{86} and GA_{87} have been detected in mature seed and seedlings, but not in flowering plants (Blake *et al.*, 2000). GA_{87} and GA_{32} have C1-2 double bonds and three or four hydroxyl groups, respectively. Unsaturated GAs with C1-2 or C2-3 double bonds were identified by Oliveira and Browning (1993) as promotory of vegetative and/or inhibitory to the reproductive growth of floral or vegetative spurs and/or the terminal shoots of *P. avium*. Generally, increased hydroxylation enhanced the efficacy of GAs with C1-2 or C2-3 double bonds to promote these effects. Bradley and Crane (1960), in addition to *P. avium*, reported GAs inhibited flowering in a number of *Prunus* spp. This effect has been observed in other woody angiosperms (e.g., *Rosa*; Roberts *et al.*, 1999). However, this phenomenon is not universal. For instance, GA_4 promoted flowering in *Malus domestica* (Looney *et al.*, 1985) and GAs often promote flowering in conifers (Ross *et al.*, 1983). This may depend on the GA applied: in *Clerodendrum thomsoniae* GA_7 promoted flowering, while GA_3 was inhibitory (Koranski *et al.*, 1979).

Mechanisms based on the physical distancing of shoot meristems from putative root derived floral inhibitors have been evoked to explain the interdependence of florogenic capacity on plant size (Hackett, 1985), and are supported by grafting studies in cherry (Oliveira and Browning, 1993). Wareing and Frydmand (1976), using *Hedera helix* as a model, proposed a possible mechanism, whereby metabolically stable GAs have a role in suppressing floral initiation during the juvenile phase, their effect diminishing as they become more diluted with tree growth. Thus, postulating their involvement in, or even the control of, the maturation process. Consequently, for certain species, GAs may promote (or maintain) the 'apparent rejuvenation' (Chapters *1* and *3*) of mature tree tissues, potentially extending the transient improvement in vigour associated with micropropagated plants ex *vitro* (Chapter *3*).

Treatment of stockplants with gibberellins has improved the adventitious rooting of cuttings taken from mature trees. The application of GA_3-impregnated lanolin paste to three year-old oak (*Quercus ithaburensis*) plants, four weeks prior to harvesting cuttings, improved bud break, shoot elongation, and increased the proportion of cuttings that rooted from less than 10, to

approximately 50 % (Eshed *et al.*, 1996). GA_3 applied by a comparable method had a similar effect on rooting recalcitrant *Citrus* cuttings, excised eight weeks after stockplants were treated (Sagee, 1990). Likewise, the rooting of avocado (*Persea americana* Mill.) cuttings was improved when donor trees were injected with GA_{4+7} three months before their excision (Cutting and Van Vuuren, 1988). Thus, GAs may assist in the propagation of elite mature trees that are frequently rooting recalcitrant. However, GAs can be inhibitory to rooting when applied just prior to the excision of cuttings, and as in Scots pine (*Pinus sylvesteris*) if applied directly after excision (Ernstsen and Hansen, 1986).

Figure 4a. Molecular structure of the *ent*-gibberellane skeleton and gibberellins A_1, A_3 and A_7 (GA_7 contains a C1-2 double bond and a single C3 hydroxyl group, whereas, GA_3 and GA_1 are di-hydroxylated (C3 and C13) with or without a C1-2 double bond, respectively)



4.1.3 Chapter aims

This chapter reports on the capacities of gibberellins A_1, A_3 and A_7, which differ in their degree of hydroxylation and saturation (Figure *4a*), to promote the vegetative growth of *ex vitro* and 'hedged' mature stockplants of *P. avium*, and the subsequent adventitious rooting of their

cuttings. That an optimal time between treatment application and excision of cuttings may exist was investigated. Additionally, exposure to the cytokinin benzyladenine was continued *ex vitro* and the effects on tree growth and the adventitious rooting capacity of cuttings were investigated.

4.2 Materials and Methods

4.2.1 Experiment 4.1: Application of GAs or BA to ex vitro P. avium plants

Experiment 4.1a: Application of treatments to ex vitro acclimatised plants

Production of rooted shoots and their transfer ex vitro

Shoot cultures of mature *P. avium* accession 1908 were initiated, maintained and rooted on ROM2 as described previously (Chapter *2*). Rooted shoots were transferred on 27 May 1997 to sterilised compost [F2 (Levington, UK), supplemented with 5 % (v/v) grit, 1 gl^{-1} (w/v) slow release fertiliser (Osmocote; Levington, UK) and autoclaved (30 min., 121 °C)] which had been treated, post-autoclaving, with a translocatable protectant carbamate fungicide (Filex; Levington, UK), in accordance with the manufacturer's instructions. Compost was contained in 175 cm^3 capacity Rootrainer pots (Ronaash Ltd, UK).

During acclimatisation of plants to *ex vitro* conditions, and treatment applications, growth-room conditions were as described previously (Section *2.2*), except that the room was maintained at 70 % relative humidity. Plants, were initially put into perspex incubators, the vents of which were closed for two days, and then opened gradually over several days to decrease humidity, until the lids could be removed without the plants wilting.

Experimental design and treatments

Plants were segregated into 15 blocks containing 6 plants of similar height. Interplant shading was reduced by placing 2 rows of 3 plants around a central row of 3 empty Rootrainer pots and a 10-12 cm gap between blocks. Within each block, plants were allocated randomly to treatments, which consisted of GA_1, GA_3, GA_7, BA, 70 % (v/v) ethanol or untreated.

Per treatment, 10 µg of GAs, 50 µg of BA (2 µg μl^{-1} and 10 µg μl^{-1} in 70 % (v/v) ethanol, respectively) or 5 µl of 70 % (v/v) ethanol (control treatment) were pipetted onto the axil of the nearest unfurled leaf to the apical meristem. Treatments were applied 4 times, at 4 d intervals, commencing on 16 June 1997. Lengths of the five internodes nearest the shoot tip, and height gain from soil level, were recorded 28 d later.

Experiment 4.1b: Application of GAs and BA to ex vitro trees grown under field conditions

Preparing trees for planting in the field trial

On 31 July 1997, plants produced in experiment *4.1a* were re-potted into 2 l volumes of fresh compost, of the composition described previously, but without fungicide or autoclaving treatments. These were maintained within a heated glasshouse and transferred, during November 1997, to a unheated polythene tunnel to harden-off in preparation for planting out the following spring. Mild weather conditions during January 1998 necessitated their transfer to a non-illuminated cold room maintained at 6-8 °C, to slow down bud break until trees could be planted at the trial site.

Planting and maintenance of the field trial

The trial site was located at Rains Brook, Barby Lane, Rugby, UK (Figure *4c*), and comprised a previously ridge and furrowed, grazed grass, field over clay. Land drains were installed in the month proceeding planting. During February 1998, planting commenced of containerised experimental, and bare-rooted [commercially obtained 400-600 mm seedling transplants (Woodland Improvements and Conservation Ltd, UK) of German origin (EEC-plant passport UK/EW20138 batch 80108704)] guard trees, at 2.5 m x 2.5 m spacing. Trees were retained in the treatment structure used *ex vitro* (experiment *4.1a*) and the distribution of treatments within blocks, and blocks across the site, were randomised. Guard trees were planted around the trial site perimeter, areas considered most prone to water logging and near the routes taken by land drains. All trees were staked and protected with 750 mm tall tree guards (Tubex Ltd, UK; Figure *4d*). Chemical weed (to 0.25 m radius around trees) and pest (mainly black-fly, *Myzus cerasi*) control management was carried out (in accordance with HRI's pest and weed control programme) each May, with further applications during the trees growth season, as required.

Treatments and data recording

Growth regulator and control treatments, as described previously in experiment *4.1a*, were reapplied commencing the 4 August 1998 and 30 June 1999 and cuttings were excised on 2 September 1998 and 1 August 1999 (29 and 32 d later, respectively). Treatments were applied to a maximum of three branches per tree and the leader shoot tip; however, only branches were

used as cuttings. Leader heights were recorded on 27 May 1998 and 21 May 1999, prior to commencing treatments, and again at the time of taking cuttings. Numbers of branches produced were recorded each May and at excision of cuttings. Branch lengths, and whether apices were in active growth, were recorded for branches used for cuttings prior to commencing treatments and before cuttings were excised. The proportions of shoots with callus, roots and the number of roots per rooted cutting were recorded 72 d after the excision of cuttings.

Preparation of cuttings

Cuttings (150 – 200 mm in length) were kept cool and moist after excision, while all but the uppermost four/six leaves were removed, the basal 10-15 mm of stem was treated with auxin [5 min. in 250 mg l^{-1} IBA dissolved in 100 % acetone, with 15 min. for the solvent to evaporate], inverted, and except the basal 2 cm of the cuttings, submerged in Benlate commercial fungicide (Dupont, UK) [0.2 % (w/v) aqueous suspension of Benlate, giving a benomyl concentration of 0.1 % (w/v)]. Cuttings were inserted individually into compost [50 % (v/v) fine bark and sphagnum moss peat, supplemented with 0.1 % (w/v) 'Osmocote' slow release fertiliser and 0.1 % (v/v) 'Intercept' fungicide] contained in 500 cm^3 capacity pots. These were randomised in blocks, consisting of one of each treatment per block, within a polythene tent (Figure *4e*), maintained at 90-100 % humidity with pulsed fog (Figure *4f*) to maintain leaf wetting, under-bench heating (22 ± 5 °C), and shaded during hot weather.

4.2.2 Experiment 4.2: Application of GA_7 to mature P. avium hedged trees

During early May 1999, twelve hedged mature *P. avium* c.v. F12/1 trees were hard-pruned and sprayed against *M. cerasi*. Branches within the hedge were allocated randomly to each treatment, with 2 blocks of neighbouring branches, containing 5 treatments, per tree, making a total of 24 branches per treatment.

Beginning on the 8 June 1999, at 30, 20, 10 or 0 d before excision of cuttings, 40 µg of GA_7 (2 µg $µl^{-1}$ in 70 % (v/v) ethanol), or 20 µl of 70 % (v/v) ethanol only at 0 d, were applied to branches using the method previously described (experiment *4.1a*). Branch lengths were recorded 30 d prior to, and at, the time of excision of cuttings. Whether the apices of branches used for cuttings were actively growing when cuttings were excised was also recorded. The

proportions of cuttings that rooted and the number of roots per rooted cutting were recorded 70 d later.

Shortly after the excision of cuttings, hedges were hard-pruned back to 2 buds of the present season's growth and re-sprayed against *M. cerasi*. A second replicate experiment commenced on the 15 September 1999.

4.2.3 Statistical analyses

Statistical analyses were carried out with Genstat V software (Genstat 5 Committee, 1993). Leader height and branch length increments were compared by analysis of variance following square root transformation. Height/length increment data were analysed with initial height as a covariate, but this was subsequently removed from the analysis since it was not significant. Internode length was analysed with ANOVA for a balanced experiment. Generalised linear models (McCullagh and Nelder, 1989) with binomial error and the logit link function were used to compare the proportions of cuttings that rooted and activity of apices. To compare the number of roots per rooted cutting, and number of branches, generalised linear models with positive poisson error and a link function of log (mean -1) were used (Ridout and Demetrios, 1992). The threshold for statistical significance was taken to be the P=0.05 probability level.

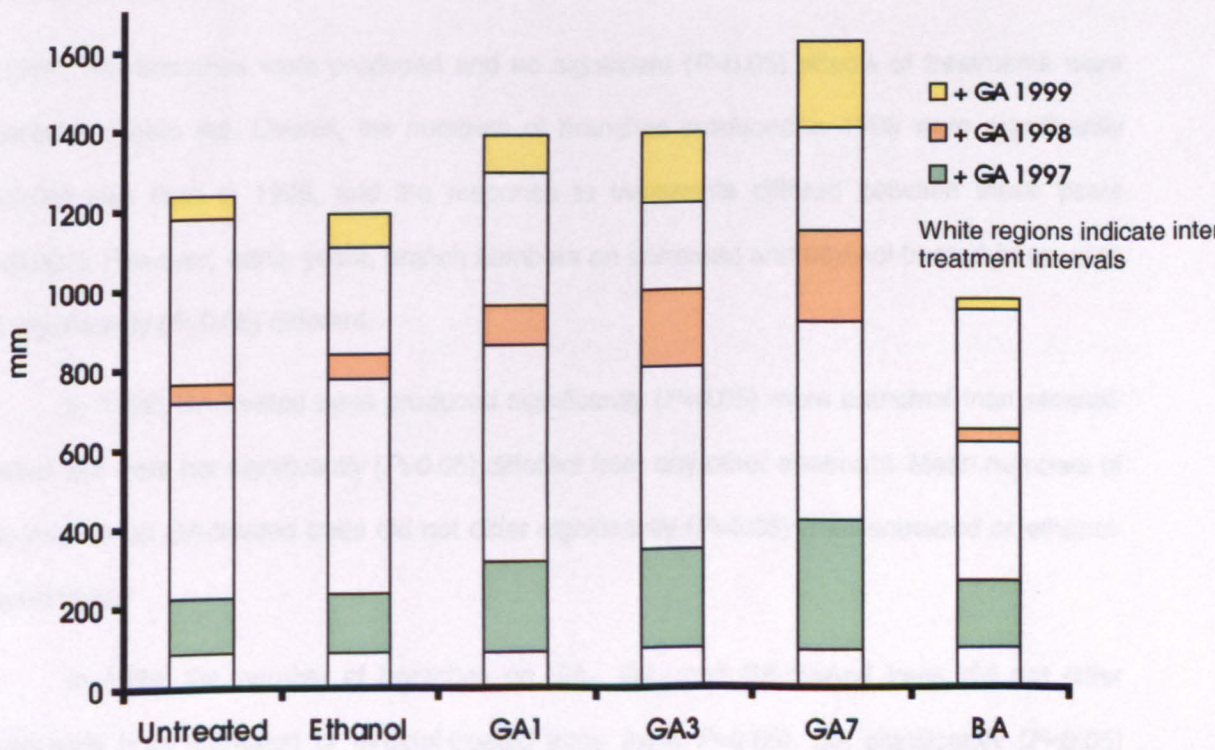
4.3 Results

4.3.1 *Experiment 4.1: Effects of GAs and BA on the growth of ex vitro P. avium trees and the rooting capacity of cuttings*

Experiment 4.1a: Height increments of ex vitro acclimatised plants during 1997

Initially, the mean heights of plants assigned to different treatments did not differ significantly (P=0.05), with a pooled mean (± SE) of 88 ± 3.3 mm. Twenty-eight days later, at the end of the first series of treatment applications (+GA, 1997; Figure *4b*), untreated and ethanol only control plants were not significantly (P=0.05) different. However, the height increment of BA-treated plants (78 mm) was just significantly (P<0.05) greater than untreated plants (55.3 mm), although, not significantly different from ethanol-only treated plants (61 mm). All GA-treated plants had greater height increments than either control or BA-treated plants (P<0.001). GA_7-treated plants had a greater height increment (235 mm) than all other treatments (P<0.001). Height increments did not differ significantly (P=0.05) between GA_1 and GA_3-treated plants.

Figure 4b. Effects of exogenous GAs and BA on mean height increments of *P. avium* accession 1908 plants after acclimation to *ex vitro* conditions, during 1997, and while grown under field trial conditions, during 1998/99



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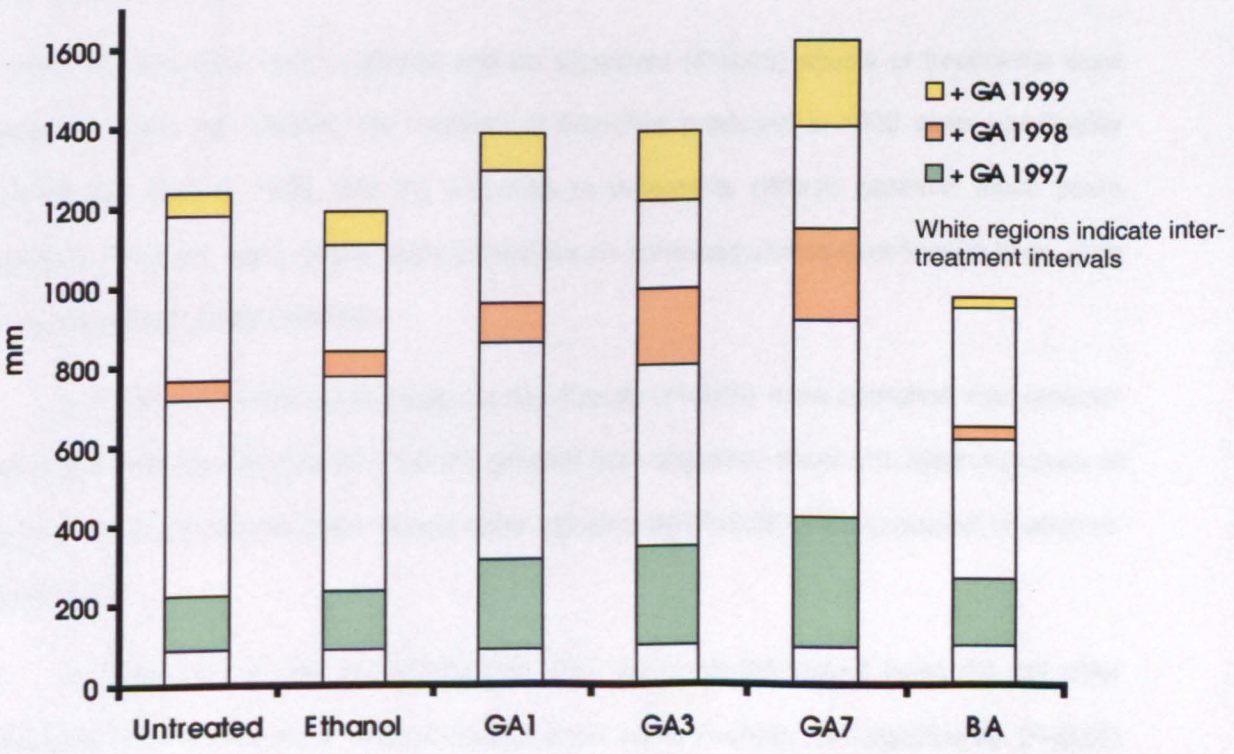


Untreated, ethanol and BA treated plants did not differ significantly in mean internode lengths (15.2, 15.4 and 13.3 mm, respectively). All GA-treated plants had significantly longer internodes than either control or BA-treated plants ($P<0.001$). GA_1 and GA_3-treated plants did not differ in internode length (24.9 and 22.9 mm, respectively), but the internodes of GA_7-treated plants were significantly ($P<0.001$) longer (28.9 mm) than those from other treatments.

Experiment 4.1b: Effects of GA and BA on the growth of ex vitro trees grown under field trial conditions in 1998 and 1999, and the subsequent rooting of cuttings

Height increments during treatment intervals

There were no significant ($P=0.05$) interactions between treatments in 1998 and 1999, hence, data were pooled for further analysis. Height increments of GA_3 and GA_7-treated trees (Figure *4b*), which did not differ significantly ($P=0.05$) from each other, were significantly ($P<0.01$) greater than all other treatments. GA_1-treated trees did not differ significantly ($P=0.05$) from ethanol-treated trees, but were significantly ($P<0.05$) greater than untreated. BA-treated trees were significantly ($P<0.05$) shorter than all GA and ethanol-treated, but were not significantly ($P=0.05$) different from untreated trees

Number of branches

In 1997, few branches were produced and no significant ($P=0.05$) effects of treatments were detectable (Table *4a*). Overall, the numbers of branches produced in 1998 were significantly ($P<0.05$) less than in 1999, and the response to treatments differed between these years ($P<0.001$). However, within years, branch numbers on untreated and ethanol-treated trees were not significantly ($P=0.05$) different.

In 1998, BA-treated trees produced significantly ($P<0.05$) more branches than ethanol-treated, but were not significantly ($P=0.05$) different from any other treatment. Mean numbers of branches on all GA-treated trees did not differ significantly ($P=0.05$) from untreated or ethanol-treated trees.

In 1999, the number of branches on GA_1, GA_3 and BA-treated trees did not differ significantly from untreated or ethanol-treated trees (both $P=0.05$), but significantly ($P<0.05$)

fewer branches were produced on GA_7-treated trees than those treated with ethanol or untreated.

Table 4a. Effects of exogenous GAs and BA on the mean number of branches produced by *ex vitro P. avium* accession 1908 trees grown under field trial conditions

Treatment	Mean number of branches				
	1997	1998		1999	
		27 May	2 September [a]	21 May	1 August [a]
Untreated	0.1	3.0	3.1	4.3	5.5
Ethanol	0	2.3	2.7	3.7	4.7
GA_1	0.1	2.9	3.0	4.0	5.7
GA_3	0	3.7	3.6	2.3	3.5
GA_7	0	3.5	4.2	2.3	2.8
BA	0.4	4.5	5.6	3.6	5.0

[a] Data were recorded just prior to the excision of cuttings

Length increments of branches used for cuttings

Overall, branches were significantly (P<0.001) shorter in 1998 than 1999. However, there were no significant (P=0.05) interaction between treatments and year, hence, data were pooled for further analysis. Mean length increments of ethanol-treated and untreated branches did not differ significantly (P<0.05;Table 4*b*). Overall, GA-treated branches had greater (P<0.001) length increments than all other treatments, with the increment for GA_7-treated branches significantly (P<0.001) greater than that of the other GAs. BA-treated branches had a significantly (P<0.05) lower growth increment than those treated with any GAs or untreated, but were not significantly (P=0.05) different from ethanol-treated.

Table 4b. Effects of exogenous GAs and BA on the length increments of branches excised for cuttings from *ex vitro P. avium* accession 1908 trees grown under field trial conditions

Treatment	Length (mm)			
	1998		1999	
	Initial 5 August	Increment at 2 September	Initial 4 July	Increment at 1 August
Untreated	341	8.4	408	31
Ethanol	395	5.9	452	18
GA_1	394	32	479	51
GA_3	384	61	562	66
GA_7	327	128	598	139
BA	286	5.2	315	6.2

Data were recorded at commencement of treatments and just prior to the excision of cuttings

Activity of branch apices at excision of cuttings

Overall, the proportions of apices active at the time of excision of cuttings did not differ significantly (P=0.05) between 1998 and 1999, but baseline activity (i.e., control treatments) was significantly (P<0.01) greater in 1999 (Table *4c*).

In 1998, all GA-treated branches had significantly (P<0.01) greater proportions of active apices compared to non-GA-treated branches. In 1999, branches treated with GA_7 and GA_3 had significantly (all P<0.05) greater proportions of apices active than ethanol-treated or untreated. GA_1, ethanol-treated and untreated were not significantly (P=0.05) different from each other, whereas, all treatments had significantly greater proportions of branches with active apices in comparison to BA-treated branches.

There was no significant (P=0.05) correlation between apex activity (Table *4c*) and the subsequent rooting capacity of cuttings (Table *4d*) derived from treated branches.

Table 4c. Influence of exogenous GAs or BA on the activity of branch apices of *ex vitro P. avium* accession 1908 mother trees, grown under field trial conditions, subsequent used for cuttings

	Treatments						
	Untreated	Ethanol	GA_1	GA_3	GA_7	BA	Totals
Number tested [a]	44	45	45	45	45	39	
Number of cuttings with actively growing apices at excision during:							
1998	2	4	12	15	19	4	
1999	9	8	14	16	18	4	
Totals	11	12	26	31	37	8	

[a] Values were derived from two replicate experiments, over consecutive years, each with sample sizes ranging from 21-23 cuttings

Rooting of cuttings

Overall, the proportions of cuttings that produced callus and roots were lower in 1998 than during 1999 ($P<0.001$). However, there were no significant ($P=0.05$) differences in response to treatments between years, hence, data were pooled for further analysis. There were no significant (both $P=0.05$) differences in the proportions of cuttings from different treatments with callus or roots (Table 4*d*). Within each year, there were significant tree-to-tree variations in the capacity of cuttings to produce callus and roots, but for individual trees this interaction was inconsistent between years.

There were no significant ($P=0.05$) effects of branch length/increment (Table *4b*) on the subsequent capacity of cuttings to produce callus and roots (Table *4d*).

The number of roots per rooted cutting (Table *4d*) did not differ significantly between cuttings taken from untreated, ethanol, GA_7 or BA-treated trees ($P=0.05$). GA_1-treated trees produced cuttings that had significantly ($P<0.05$) fewer roots than ethanol-treated, but did not differ significantly ($P=0.05$) from untreated. Data suggests cuttings from GA_3-treated trees may have produced fewer roots than those from other treatments but the low number of cuttings that rooted make confidence uncertain.

Table 4d. The proportions of cuttings producing callus or roots and the number of roots per rooted shoot influence by exogenous GAs or BA application to *ex vitro P. avium* accession 1908 mother trees, grown under field trial conditions

	Treatments					
	Untreated	Ethanol	GA_1	GA_3	GA_7	BA
Number tested [a]	44	45	45	45	45	39
Proportions (%) of cuttings with:						
Callus	64	63	76	67	62	57
Roots	48.9	37	42	31	40	42.9
Number of roots per rooted cutting	3.45	3.9	2.75	2.75	3.75	4.8

[a] Values were derived from two replicate experiments, over consecutive years, each with sample sizes ranging from 21-23 cuttings

4.3.2 Experiment 4.2: Effect of GA_7 application to mature P. avium cv. F12/1 hedged trees on branch growth and the rooting capacity of cuttings

Length increments of branches used for cuttings

There were no significant (P=0.05) interaction between treatment and replicate experiments, hence, data were pooled for further analysis. Overall, the initial mean length of branches was significantly (P<0.001) greater in July than September (Table *4e*), as was mean length increment. (P<0.001).

Branches ethanol-treated at 0 d, and GA_7-treated branches from 0 or 10 d prior to the excision of cuttings did not differ significantly (P=0.05) from each other in length increments. However, branches GA_7-treated at 20 or 30 d prior to the excision of cuttings, had significantly (P<0.001) greater length increments than ethanol-treated at 0 d, with those treated at 30 d prior to excision having the greater increment.

Table 4e. Effects of exogenous GA_7 on branch length increment and the activity of apices prior to the excision of cuttings from mature hedged *P. avium* cv. F12/1, during 1999

		Mean increments for individual treatments (mm)				
		Number of days prior to excision of cuttings that treatment were applied				
Combined data For both experiments		+GA_7				Ethanol
		-30	-20	-10	0	0
		201	165	146	128	122
		(14.2)	(12.8)	(12.1)	(11.3)	(11)
		SED. [a]	(0.88)			
		d.f. 227				
		Proportions (%) of cuttings with active apices at excision				
1	9 July	88	75	74	71	87
2	15 October	92	65	21	13	4

[a] Standard error of difference (SED) are for comparison between means of square transformed data, presented in parentheses
Values for each experiment were derived from 24 shoots per treatment

Activity of branch apices at excision of cuttings

Overall, there were significant (P<0.001) differences in response to treatments between July and October (Table *4e*). In July, there were no significant (P=0.05) differences between treatments in the proportions of apices that were active at the excision of cuttings. Similarly, in October, there were no significant (P=0.05) differences in the activities of apices on branches treated with ethanol-only at 0 d and GA_7-treated at 0 or 10 d prior to taking cuttings. Conversely, GA_7-treated branches at 20 and 30 d prior to the excision of cuttings had significantly (P<0.01 and P<0.001, respectively) greater proportions of active apices, compared with ethanol-treated at 0 d.

Rooting of cuttings

There were no significant (P=0.05) differences in response to treatments between replicate experiments, hence, data were pooled for further analysis. There were no significant differences between treatments in the proportions of cuttings that produced basal callus (P=0.05; Table *4f*). Likewise, the proportions of cuttings that rooted were not significantly (P=0.05) different for any

treatment where GA_7 had been applied. However, the proportions of cuttings that rooted from all GA treated branches were significantly (P=0.05) greater than cuttings from ethanol only treated branches. However, there was no correlation between the number of apices active and the proportions of cuttings that rooted (P=0.05).

Table 4f. Effects of the timing of exogenous GA_7 application to branches of mature hedged *P. avium* cv. F12/1on the subsequent rooting capacity of these branches as cuttings

	Number of days prior to excising cuttings that GAs were applied				
	+GA				Ethanol
	-30	-20	-10	0	0
Proportions [a] (%) with:					
Callus	91	88	83	88	79
Roots	57	50	50	50	29
Number of roots per rooted shoot	3.2	2.8	3.8	3.4	2.4

[a] Values were derived from 24 shoots per treatment

Figures 4c-h. Rains Brook field trial site and the rooting of GA or BA-treated cuttings

Figure key:

c. Rains Brook field trial site

d. Cherry tree during 1st year after planting

e. Fogging unit with cuttings

f. Interior of unit with fog burst in progress

g. Cherry cuttings after 70 d in the unit

h. Rooted and non-rooted (with basal callus) cherry cuttings after 70 d in the unit

Scale bars = 100 mm

4.4 Discussion

Mature woody perennials are typically more difficult to propagate vegetatively than their juvenile counterparts, implying developmental phase is significant factor acting upon rooting success (Section *1.3*). GAs can improve the rooting of cuttings from mature trees (Eshed *et al.*,1996). GA content/concentration differs between the development phases of cherry (Blake *et al.*, 2000; Section *4.1.2*), and exogenous GAs with structural analogy to those found in abundance in the juvenile phase can promote the development of juvenile-like characteristics in mature tissues, e.g., floral inhibition, increased vegetative growth and altered phyllotaxy [Oliveira and Browning (1993a/b), Hackett (1985)]. The ability of GAs to manipulate phase was further demonstrated by the complete inhibition of floral initiation when mature cherry scions were grafted onto seedlings, and in reciprocal experiments, when florogenic activity in juvenile scions was promoted (Oliveira and Browning, 1993b). Thus, the involvement of GAs in (or even controlling) the maturation process has been postulated (Wareing and Frydmand,1976). This chapter tested the efficacy of GAs to promote rooting in hedged mature *P. avium* trees and (or maintain) the 'apparent rejuvenation' of trees ex *vitro* (Chapter *3*). Hence, the primary hypotheses tested whether exogenous GAs can improve the rooting of *P. avium* cuttings, and that efficacy to promote rooting would follow predicted structure-activity relationships.

Similarly to GAs, cytokinins have been shown to affect floral initiation (Bernier *et al.*, 1990). During the micropropagation of *P. avium*, cytokinin is used to promote shoot proliferation (Chapter *3*). Thus, an association may exist between the use of cytokinins in a process that is postulated to result in the 'apparent rejuvenation' of mature tissues, and capacity to modify florogenic activity (the ultimate marker of maturation). Hence, the supply of cytokinin (BA) was continued *ex vitro* to investigate possible effects on rooting. BA was chosen because it had been used previously during the micropropagation of *P. avium* (Chapters *2* and *3*).

Unsaturated GAs with C1-2 or C2-3 double bonds were identified by Oliveira and Browning (1993) as promotory of vegetative and/or inhibitory to the reproductive growth of seedling and mature *P. avium*. A number of 12α, 13-hydroxy GAs have been detected in mature seed and seedlings of *P. avium*, but not in flowering plants (Blake *et al.*, 2000), and two, GA_{87}

and GA_{32}, are highly active (Blake *et al.*, 2000), as predicted by Oliveira and Browning (1993). However, these GAs are not freely available, so, alternatives were sought with which to test the structure-activity relationship on *ex vitro* and mature cherry. Predicted, structure-activity would rank these as GA_3 > GA_7 > and GA_1, in order of efficacy to promote vegetative growth and/or inhibit florogenic activity (GA_7 contains a C1-2 double bond and a single C3 hydroxyl group, whereas, GA_3 and GA_1 are di-hydroxylated (C3 and C13) with or without a C1-2 double bond, respectively).

4.4.1 Treatment effects on ex vitro trees: growth and rooting

Leader/branch extension, number of branches and activity of apices provided means to measure whether GA and BA treatments were having physiological activity, and as such were supplementary to investigating the effects of GAs on rooting. However, they provided interesting additional results.

All GAs were active at promoting leader height gain on *P. avium* plants when applied shortly after acclimatisation *ex vitro* in 1997; GA_7 was the most promotory. However, GA_1 was less effective at eliciting a response on trees grown under field trial conditions, GA_3 and GA_7 did not differ significantly. Nevertheless, by the end of this study, GA_7-treated trees were taller than those from other treatments. That GA_1 was less effective than other GAs at promoting vegetative growth is consistent with predicted activity, but the activity of GA_7 over that of GA_3 is counter to the activity model proposed by Oliveira and Browning (1993). However they noted that GA_9 (without a C1-2 or C2-3 double bond or hydroxyl groups) and GA_7 were equally as effective at promoting shoot growth of 1-year-old *P. avium* seedlings (unfortunately, GA_3 activity was not reported), but GA_9 failed to inhibit floral initiation. Similarly with *Prunus cerasus*, GA_9 inhibited floral initiation and GA_7 and GA_4 were as effective as each other (Burkovac and Yuda, 1991). However, treatment of trees under field trial conditions suggests that the activity of GA_7, which differs from GA_3 by having one less hydroxyl group, was inconsistent with that predicted for mature *P. avium*. Thus, data indicates that plants recently acclimatised *ex vitro* behaved similarly to seedlings treated by Oliveira and Browning (1993), and that this effect was carried over into the field. This is in agreement with the hypothesis that micropropagation promotes the 'apparent rejuvenation' of mature trees (Chapter *3*).

GA_7-treated branches elongated more than any other treatment. Branch number showed no consist effect of treatments from year-to-year, but there was an overall trend of a year-on-year increase. Nevertheless, in 1999, GA_7-treated trees had fewer branches than those given other treatments. Further data to determine if this effect continued would have been useful. Hammatt (1999), reported that removal of branches form seedling derived *P. avium* trees promoted height gain, possibly, as a result of reducing competition with the leader shoot. It may be interesting to investigate the potential of GA_7 in control of tree form. GA_7 and GA_3 maintained apical activity which may have silvicultural utility in extending the growing season. The latter effect fits the known seasonal fluctuations in the GA content of *P. avium*, and possible other broadleaves. Overall, the endogenous concentrations of GAs in *P. avium*, are reportedly higher during the spring and early summer, while shoots are actively growing, the decline of which correlates with termination of vegetative growth and the initiation of florogenic activity in competent tissues (Blake *et al.*, 2000).

Rooting of cuttings

In a number of species, adventitious roots develop indirectly from cuttings, via a callus phase (Section *1.2.1*), and this occurs with cherry. There were no significant effects of any treatment on callus or root formation (confidence is uncertain, due to a small sample of cuttings that rooted, cuttings from GA_3-treated trees may have had fewer roots). Although, no without auxin control was included, formation of callus implies that cuttings were capable of perceiving the auxin root-inducing stimuli and that only a proportion of these were competent (Section *1.5.1*) to become determined and differentiate to form roots (that emerged). Hence, on basis of this data, the hypothesis that GAs would promote the rooting of *P. avium* cuttings, derived from trees of *ex vitro* origin, is rejected.

4.4.2 Treatment effects on hedged mature trees: growth and rooting

Mature cuttings were taken from branches of *P. avium* cv. F12/1 scions that had been grafted onto cv. Colt rootstock and maintained as a field-grown hedge, with hard pruning annually each spring (Section *2.1*). Evidence suggesting that this hedge is mature includes: florogenic competence and reproducible initial sub-optimal rooting when shoots are micropropagated, with a progressive increase in rooting capacity with time *in vitro* (Hammatt and Grant, 1993, 1997a),

which can be sustained, along with transient inhibition of flowering, on transfer *ex vitro* (Hammatt, 1999). Generally, cuttings from hedged stockplants root more easily than counterparts from unpruned mature trees, implying that for rooting, the hedge (or at least the subtending stems) may behave as if semi-mature.

Instead of GA_3 (the predicted most active GA), GA_7 which had been shown to be physiologically active, at least, in promoting vegetative growth in the trial with ex vitro trees at Rains Brook, when applied to shoot tips of the (putatively) mature hedge. Unsurprisingly, GA_7-treated branch elongation correlated positively with increasing time between GA_7 application and excision of cuttings. Apical activity when cuttings were excised was unaffected by treatment in July, but showed a similar positive correlation with increasing time between GA_7 application and excision of cuttings.

The time interval between GA application and taking cuttings varies significantly between published reports (Section *4.1.2*). Eshed *et al.* (1996) reported in *Quercus ithaburensis*, that GA_3 inhibited rooting if applied to the base of cuttings just after excision but was promotory if 4 weeks had elapsed between application and the subsequent excision of cuttings. That an optimal time interval may exist between these activities was investigated in this study with *P. avium*.

Rooting

There were no effects of treatment on the proportions of cuttings that produced callus. Where GA_7 had been applied, there were no significant differences in the proportions of cuttings that rooted, but non-GA-treated trees produced cuttings that rooted less easily. Overall, a pooled proportion of 52 % of GA_7-treated branches rooted compared with 29 % for non-GA-treated trees. This improvement was not attributable to any GA-induced structural changes to the stem prior to excising the cutting [e.g., GAs may regulate the orientation of cellulose microtubules, hence, orientation of cell wall cellulose (Hooley, 1994)], but does not preclude any post-excision changes. That differences also occurred between GA_7-treated and untreated branches where cuttings were treated on the day of excision appears inconsistent with the inhibitory effect on rooting reported by Eshed *et al.* (1996). However, the treatment application sites differed between these experiments, maybe, target(s) site(s) of action differed and/or transported GA_7

was modified *in planta* before cutting base. No correlation existed between apical activity and the subsequent rooting of cuttings.

This result suggests that GA_7 increased the rooting of cuttings derived from hedged mature trees, supporting the original hypothesis, but is inconsistent with data from *ex vitro* trees, where no GA treatment improved rooting.

Evidence (cited above) suggests hedged *P. avium* cv. F12/1 was mature, whereas, the *ex vitro P. avium* accession 1908, had undergone full 'apparent rejuvenation' during micropropagation, as determined by rooting performance *in vitro* (Hammatt and Grant, 1997a). Therefore, the genetic rooting potential (i.e., genetically-determined maximum rooting competence) of cuttings from *ex vitro P. avium* is more likely to be near this maximum than mature hedged trees. Thus, if GAs were manipulating development phase (or rooting independently), the greater opportunity to do this may be in the hedged mature trees.

The mechanism(s) involved in the regulation of phase change (Chapters *1* and *2*) remain to be elucidated. Exogenous GA_3 has been shown to increase IAA biosynthesis (Law and Hamilton, 1985) and the rate of polar auxin transport (Basler and Mc Bride, 1977), factors that could be considered promotory of adventitious rooting.

Albeit, within the constraints of the selected GA concentrations and the putative status of mature hedged trees, differences in rooting between cuttings from both sources, and the relationships between structure-activity profile of GAs, suggests that *ex vitro P. avium* trees responded as if juvenile, which would be consistent with the hypothesis that prolonged micropropagation can promote the 'apparent rejuvenation' of mature trees (Chapter *3*).

To characterise the maturity status (or otherwise) of trees from hedged and *ex vitro* sources may be aided by the development of markers of juvenility/maturity (Chapter *3*), for example, IAA:ABA ratio (Oliveira and Browning, 1993) or a 23 kDa protein (Hand *et al.*, in progress; Section *3.1.3*), both of which have been identified in cherry.

4.4.3 Conclusions

P. avium usually begins to initiate floral buds during their fourth growing season after germination and flower the following spring. Hence, the duration of this study was insufficient to permit flower

initiation in the micropropagated plants, but it would be worth recording when this begins. Transient retardation of flowering of trees *ex vitro* has been discussed previously (Chapter *3*). To increase confidence in rooting data it would be necessary to repeat experiments. Nevertheless, this study has identified several potential key points from the use of GAs:

- GA_7 promoted rooting of cuttings from hedged mature *P. avium*, but not *ex vitro* trees.
- Rooting was not influenced by the interval between apical GA_7 application and excision of cuttings.
- GA structure-activity, predicted by the model of Oliveira and Browning (1993), within the constraints of GA concentration used and hedge maturity status, suggest that *ex vitro* trees were behaving as if juvenile.
- After two years in the field, the mean height of GA_7-treated trees were 33 % greater than untreated trees (less expensive GA_3 was also promotory) which may accelerate production of the first 4 m of trunk (the standard length required in modern timber processing).
- GA_7 maintained apices activity on *ex vitro* and mature trees which may have silvicultural utility by extending the apical growing season.

Commercially, lanolin-based methods [Eshed *et al.* (1996), Sagee *et al.* (1990)] appear possibly the most suitable mean of GA delivery, as the method used in this study would be inappropriate in forestry practice, and a spray-based application potentially environmentally polluting. In caution, although unlikely, extending the growing season may make shoot apices more liable to frost damage, should growth continue into autumn. Furthermore, this study did not evaluate the effects of GA on root architecture: possibly, this may be discordant if above ground growth was at the expense of root system development. Indeed, the assumption that the root architecture of established trees derived from micropropagated and conventionally propagated sources are similar has not been investigated for any species to date.

5. Adventitious Shoot Regeneration from Wild Cherry (*Prunus avium* L.) Leaves

(Results published in: Grant and Hammatt, 2000; a copy of which is appended)

5.1 Introduction

Genetic improvement of most tree species by conventional means is hindered by breeding systems based on obligate out-crossing and extended juvenile periods of 4-30 years. Thus, this study explores genetic transformation as a complementary method to manipulate a genetic trait, that of adventitious rooting. *P. avium* is a species which has potential as a high value tree and merit as a woody model (discussed in Section *1.5.2*).

A regeneration method using either adventitious shoots (and subsequent rhizogenesis to produce whole plants) or somatic embryogenesis is a pre-requisite for recovering transgenic plants from transformed cells. Frequently, the regeneration of whole plants from transformed cells is a limiting factor for genetic engineering and restricts the technique to receptive species and/or genotypes, rather than those most appropriate.

There are several reports on regeneration of adventitious shoots from the *Prunus* genus [e.g., James *et al.* (1984), Ochatt and Power (1988), Ochatt (1990, 1991), Yang and Schmidt (1992), Escalettes and Dosba (1993), da Camara Machado *et al.* (1995)]. To date, only Yang and Schmidt (1992) and Hammatt and Grant (1998) have reported regeneration of adventitious shoot from *Prunus avium* leaves, and Ochatt (1991) produced shoots from protoplasts, but these studies were limited to a few genotypes and achieved only relatively low frequencies of regeneration.

5.1.1 Chapter aims

As a possible target species for transformation with the *AtAUX1* gene, to manipulate adventitious rooting capacity, this chapter reports improved methodology for adventitious shoot regeneration from *P. avium* leaves, over that previously reported by Hammatt and Grant (1998), the optimal of which gave a mean (± s.e.) of 0.7 ± 0.05 adventitious shoots from 62 % of accession 1908 leaf explants.

5.2 Materials and Methods

5.2.1 Plant materials

Prunus shoot cultures were initiated and maintained as described previously (Chapter *2*), and acted as a source of leaf explants. Furled leaves (3-5 mm long), proximal to shoot apices, were excised, cut three times across their midrib with the final cut removing the distal leaf tip and positioned with their abaxial surface in contact with regeneration medium.

5.2.2 Regeneration medium

The basic shoot regeneration medium (REM1) consisted of growth-regulator-free Woody Plant Medium (WPM; Lloyd and McCown, 1981) with 0.54 µM 1-naphthaleneacetic acid (NAA; Sigma, Dorset, UK), and 4.4 µM 1-phenyl-3-(1,2,3,-thiadiazol-5yl) urea (TDZ; Duchefa, Haarlem, NL). This medium (with or without the supplements tested in individual experiments) was dispensed as 25 ml volumes into 9 cm Petri Dishes (PDs). For all media, agar concentration, pH adjustment, autoclaving, and culturing conditions were as previously described (Section *2.2*).

5.2.3 Experiment 5.1: Genotype

Twenty-five leaf explants from each of 10 genotypes (accessions 1904, 1905, 1906, 1908, 1909, 1912, 1919, 2474, and the rootstocks F12/1 and Charger) were distributed equally amongst five 9 cm PDs containing REM1. Data were recorded 28 d after culture initiation, and cultures which did not produce shoots were transferred to fresh medium of a similar composition for a further 28 d culture period and assessed again. Experiments were carried out during November and December 1996.

5.2.4 Experiment 5.2: Wounding frequency

Per treatment, 25 leaf explants from accessions 1905 and 1908, with either 2 or 4 cuts through their midrib, were distributed equally amongst five 9 cm PDs containing REM1. Data were recorded after 21 d of culture, during April 1997.

5.2.5 Experiment 5.3: Surfactants

Per treatment, 25 leaf explants of accession 1905 were distributed equally amongst five 9 cm PDs containing REM1 supplemented with 0, 10, 100 or 1000 mg l^{-1} of the non-ionic co-polymer surfactants Tween-20, F68 or F127 (all from Sigma, Dorset, UK). Surfactants were added to

post-autoclaved medium as filter-sterilised solutions. Data were recorded after 21 d of culture, during May 1997.

5.2.6 Experiment 5.4: Silver nitrate

Per treatment, 25 leaf explants of accession 1905 were distributed equally amongst five 9 cm PDs containing REM1 supplemented with either 0, 10, 20, 40 or 80 μM silver nitrate (Sigma, Dorset, UK). Silver nitrate was added to post-autoclaved medium as filter-sterilised solution. Data were recorded after 21 d of culture, during June 1997.

5.2.7 Experimental designs

All experiment were randomised in blocks, containing one dish of each treatment per block, and repeated twice.

5.2.8 Statistical analyses

Statistical analyses were carried out with Genstat 5 software (Genstat 5 Committee, 1993). Generalised linear models (McCullagh and Nelder, 1989) and binomial error and the logit link function were used to assess whether genotype wound number or surfactants influenced the proportions of leaves with shoots. The normal approximation to the binomial (z) was used for comparing the proportions of leaves from two different accessions that regenerated. Effects of genotype and surfactants on mean number of shoots were compared by analysis of variance following square root transformation. Effects of wound number on mean number of shoots was tested using a GLM with poisson error and log link function. It was impractical to test the effects of all factors in one single multi-factorial experiment; instead, each was tested singly. Consequently, to take account of possible variability between experiments in mean shoot production by the control treatment, null hypotheses about the effects of individual treatment terms were only accepted or rejected if the replicate x treatment interaction term was not significant in the analysis. The threshold for statistical significance was taken to be the P=0.05 probability level.

5.3 Results

5.3.1 Experiment 5.1: Shoot formation from leaves of different genotypes

Expansion of furled leaves and the development of dark red nodular tissues at or near the cut surfaces of the midrib or petiole occurred rapidly within the first 7 d of culture. The emergence of adventitious shoots began after 7-10 d of culture and occurred mostly at or near the cut surfaces of the midrib or petiole from these nodular tissues, or directly from reddened areas of the leaf with minimal intervening callus (Figure *5a*).

Capacity to form adventitious shoots varied between genotypes. Relative to accession 1908, the mean number of shoots regenerating per leaf was either lower or not significantly different for seven of the ten genotypes assessed (Table *5a*). The proportion of leaves with adventitious shoots was just significantly greater for 1905 ($P<0.05$, $z= -1.98$) and 1906 ($P<0.05$, $z= -2.2$), but there was no significant difference between these accessions. Furthermore, accessions 1905 and 1906 produced significantly more shoots per leaf (both $P<0.01$; means of square-root transformed data = 1.26) from a greater number of locations per leaf (both $P<0.01$; means of square-root transformed data = 1.0 and 1.1, respectively) than accession 1908 (means of square-root transformed data for number of shoots = 0.84 with a mean number of locations per leaf = 0.74). Callus or leaves, which had not developed shoots after transfer to fresh medium of the same composition, did not produce any shoots.

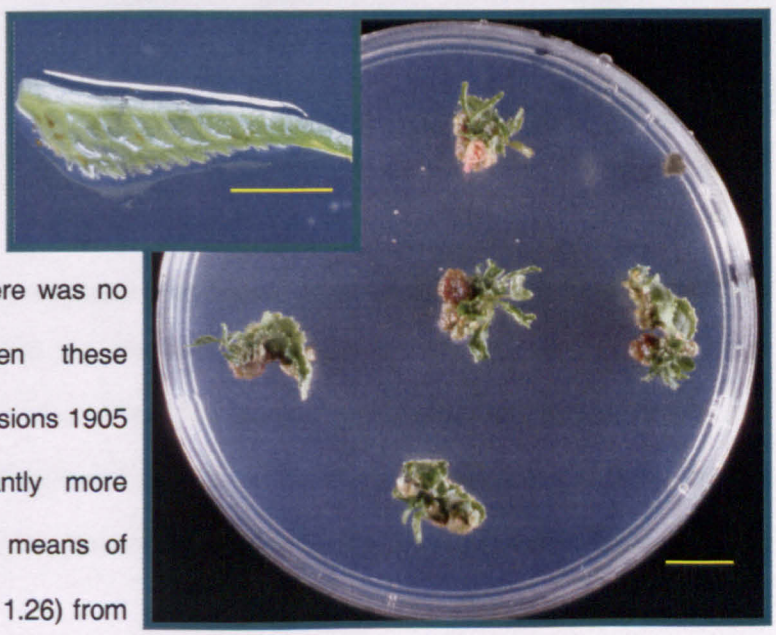


Figure 5a. Adventitious shoot regeneration from *P. avium* leaves on REM1 after 28 d of culture (scale bar = 10 mm), and insert of furled leaf explant (scale bar = 1 mm)

Table 5a. Effect of genotype on capacity to form adventitious shoots from *Prunus avium* leaves

Accession	Proportions of leaves with shoots (%)	Mean number of shoots per leaf (for all leaves)	Mean number of locations per leaf which gave rise to shoots
1908	62 ± 3.3	0.84	0.74
1904	12 ± 1.5	0.14	0.13
1905	80 ± 1.4	1.26	1.00
1906	84 ± 1.9	1.26	1.10
1909	32 ± 3.1	0.43	0.28
1912	32 ± 3.1	0.42	0.34
1919	52 ± 3.5	0.73	0.56
2474	38 ± 3.3	0.45	0.40
F12/1	50 ± 3.5	0.65	0.53
Charger	66 ± 3.4	0.95	0.75
LSD. (86 d.f.)			
5 %		0.31	0.24
1		0.41	0.33
0.1		0.54	0.44

Mean numbers of shoots and regeneration locations per leaf are for square-root transformed data
Values were derived from two experiments of 25 replicates per accession (± SE)
Data were recorded 28d after excision of leaves

5.3.2 Experiment 5.2: Shoot formation from leaves with two or four cuts to their midrib

With accessions 1905 and 1908 the formation of adventitious shoots was not improved significantly by increasing the number of cuts to the leaf midrib from two to four (Table *5b*). Although not quantified, callus appeared to form in similar quantities from each of the cut surfaces. Hence, leaves that were wounded four times may have produced relatively more callus.

Table 5b. Effect of wound number to the midrib of leaves of *Prunus avium* accession 1905 on capacity to form adventitious shoots

Number of wounds per leaf	Proportions of leaves with shoots (%)		Mean number of shoots per leaf	
	Accession			
	1905	1908	1905	1908
2	76 ± 2.58	70 ± 2.97	1.98 ± 0.21	1.58 ± 0.20
4	64 ± 3.26	66 ± 3.17	1.60 ± 0.19	1.48 ± 0.20

Mean numbers of shoots and regeneration locations per leaf are for square-root transformed data
Values were derived from two experiments of 25 replicates per treatment (± SE)
Data were recorded 21d after excision of leaves

5.3.3 Experiment 5.3: Shoot formation from leaves on medium with surfactants

Overall, response to surfactants did not differ between replicate experiments, thus data were pooled for further analyses. There were significant differences between surfactants overall ($P<0.001$). Surfactant did not influence significantly the proportion of leaves forming adventitious shoots (Table *5c*), but did have a significant effect on both shoot number and locations per leaf producing shoots.

Mean number of shoots per leaf

Relative to the control value of 2.2 shoots per leaf, Tween-20 supplemented leaves produced significantly more shoots per leaf, mean = 3.54 ($P<0.01$) and 3.16 ($P<0.05$) for 10 mg l^{-1} and 1000 mg l^{-1} surfactant, respectively (Table *5c*). Tween-20 at 100 mg l^{-1} did not influence shoot production. Other surfactants did not improve regeneration competence above that of the control; however, within these treatments, shoot number declined with increasing concentrations of F68 and F127 ($P=0.01$).

Mean number of locations per leaf where shoots formed

Overall, shoots formed in more locations on medium supplemented with surfactant than without (Table *5c*). For Tween-20 given at 10 and 1000 mg l^{-1}, the mean numbers of locations were significantly greater than the control ($P=0.05$). Similarly, this occurred with 10 mg l^{-1} F68 ($P<0.05$). There was no significant effect of increasing concentration with Tween-20. However, a linear decline with increasing F68 ($P=0.054$) and F127 ($P=0.003$) could be established.

5.3.4 Experiment 5.4: Shoot formation from leaves on medium with silver nitrate

Supplementing REM1 with silver nitrate, at the concentrations assessed, did not significantly affect shoot regeneration from accession 1905. Pooled data for the proportions (± se.) of leaves producing shoots were 79 ± 2.34 %; mean number of shoots per leaf were 2.11 ± 0.22, and the number of locations per leaf where shoots formed was 1.65 ± 0.16.

Table 5c. Effect of surfactant on the adventitious shoot regeneration capacity of *Prunus avium* accession 1905 leaves

Surfactant ($mg\ l^{-1}$)	Proportions of leaves with shoots (%)	Mean number of shoots per leaf (for all leaves)	Mean number of locations per leaf which gave rise to shoots
None	70 ± 2.97	2.20	1.54
Tween-20			
10	84 ± 1.90	3.54	2.02
100	80 ± 2.26	2.66	1.78
1000	84 ± 1.90	3.16	2.04
LSD 5% (27 d.f.)[a]		0.9	0.5
F68			
10	86 ± 1.70	2.78	1.98
100	78 ± 2.43	2.26	1.52
1000	72 ± 2.85	1.66	1.32
LSD 5% (27 d.f.)[a]		0.8	0.4
F127			
10	80 ± 2.26	2.54	1.76
100	78 ± 2.43	2.40	1.58
1000	70 ± 2.97	1.48	1.08
LSD 5% (27 d.f.)[a]		0.7	0.4
(72 d.f.)[b]		0.8	0.44

[a] LSDs for comparison between surfactant and no surfactant control.
[b] LSDs for comparison between all treatments.
Mean numbers of shoots and regeneration locations per leaf are for square-root transformed data
Values were derived from two experiments of 25 replicates per treatment (± SE).
Data were recorded 21d after excision of leaves.

5.4 Discussion

These results resolve further conditions required to promote adventitious shoots to form from *P. avium* leaves, building on findings previously published by Hammatt and Grant (1998) and on the significance of explant size/maturity. Small (3-5 mm long) furled leaves of accession 1908 had greater competence to form adventitious shoots than either very small (< 3 mm) or larger unfurled (5-10 mm) leaves [data collected just prior to beginning this thesis and published with the results of this Chapter; Grant and Hammatt (2000), a pre-print of which is appended (Section *10.5*)]. Ideally, transformation and regeneration protocols should be applicable to as wide a range of genotypes of any given species as possible. These data suggest that the basic regeneration protocol published by Hammatt and Grant (1998) could be applied to a wider range of *P. avium* accessions from British woodlands, but regeneration competence was accession-dependent, as found by Yang and Schmidt (1992) for sweet cherry. Interestingly, differences in the number of axillary shoots produced by shoot cultures of these British woodlands accessions have also been reported (Hammatt and Grant, 1997).

Significantly, failure to induce adventitious shoot regeneration other than directly from the initial explant limited shoot regeneration competence to a very narrow window of opportunity. This problem was compounded by the comparatively low mean number of shoots that formed per leaf. In transformation experiments, these factors may impede the selection and recovery of transgenic shoots. Delaying the application of selection could also increase the likelihood of transformed/non-transformed chimaeric plants being produced. Thus, frequency of wounding, surfactant and silver nitrate supplements were investigated as methods to increase the mean number of shoots that formed per leaf, but only surfactant treatment had any significant effect.

Adventitious shoot regeneration of *P. avium* was predominantly associated with sites of wounding. Brand and Lineberger (1988) reported that adventitious shoot formation of mature phase sweet gum, *Liquidamber styraciflua* var. 'Mariane', was promoted by increasing the number of wounds across the lamina and midrib of leaf explants. However, this was not successful with a second variety (Variegata), perhaps as a result of having an inherently higher organogenic competence. Hence, the rationale pursued with *P. avium*, but no improvement was observed. In explanation, wounding such small leaves (3-5 mm long) with four cuts may have

caused excessive damage (e.g., by desiccation, enzymatic degradation, release of phenolics, or volatiles such as ethanol and ethylene) to the surrounding tissues. Thus, cellular competence to regenerate shoots may have been inhibited or led to the necrosis of competent cells before shoot primordia developed. Alternatively, although not quantified, callus appeared to develop equally from each cut surface, hence leaves with greater wounding produced relatively more callus. This may have diverted nutrients away from competent cells or altered the growth regulator concentrations locally inhibiting shoot regeneration.

Non-ionic surfactants are frequently used in animal cell culture to reduce membrane damage caused during forced aeration. *In planta* they are postulated to increase the permeability of the plasma membrane to growth regulators and/or nutrients by creating transient increases in membrane fluidity (Lowe, *et al.* 1994). They have improved adventitious shoot regeneration in a number of species [e.g., *Corchorus capsularis*, Khatun *et al.* (1993); and *Populus spp.*, Iordan-Costache *et al.* (1995)].

In this study with *P. avium* accession 1905, Tween-20 improved the number of shoots produced per leaf and the number of positions per leaf that formed shoots, but not the proportion of leaves with shoots. This promotory effect was most significant at the lowest concentration assessed (10 mg l^{-1}). Khatun *et al.* (1993) reported Tween-20 to be most effective at stimulating shoot regeneration from jute cotyledons at between 10 and 100 mg l^{-1}, with an optimal response at the lowest concentration assessed (10 mg l^{-1}), and was inhibitory at 5000 mg l^{-1}. Conceivably, with both species, better regeneration frequencies may have been achieved by reducing surfactant concentration below 10 mg l^{-1}. Relative to the effect with *P. avium* accession 1905, the superior promotory response reported by Khatun *et al.* (1993) may have resulted from the use of cotyledon tissues, which are frequently more responsive than comparatively mature leaves. Nevertheless, Iordan-Costache *et al.* (1995) found a low concentration of the non-ionic surfactant Pluronic F68 improved shoot regeneration from relatively mature tissues (i.e., stem and leaf explants) of *Populus* species. However, their results showed a response which was partially genotype-dependent, and within genotype, related to the type of explant. Thus, the lower level of response observed with cherry may reflect similar factors.

The physiological effect of surfactants has been postulated to be, at least, partially dependent on a molecule's hydrophilic-lipophilic balance (HLB). A low HLB index, an arbitrary

empirical indicator of their emulsification potential (Khatun *et al.*,1993), is indicative of a surfactant with a higher hydrophobic component, and therefore, potentially greater effectiveness at causing plasma membrane solubilisation. That Tween-20 (HLB 16.7) should give a better response than either Pluronics F127 (HLB 22.0) or F68 (HLB 29.0) is interesting, but should be considered with caution as these surfactants differ in their average molecular mass (1225, 1260 and 8350, respectively), and were not compared on a molecular basis. A similar relationship between HLB and potency to promote adventitious regeneration was observed by Khatun *et al.* (1993), but again, surfactants were not compared on a molecular basis.

Results suggest that there was a strong genetic effect on shoot regeneration competence in this species. The data from surfactants is promising, but should be evaluated on a wider range of genotypes.

Many studies associate ethylene with recalcitrance in caulogenesis and adventitious shoot regeneration, although there are many contradictory reports. The effect(s) of ethylene are dependant on concentration and/or species (Biddington, 1992); and in *Garcinia mangostana* at least, the organogenic development state of the explant at the time of application (Lakshmanan *et al.*, 1997). Thus, the effect of ethylene for a given situation often requires determination. Perhaps, the most direct evidence linking ethylene with the inhibition of shoot regeneration is where an anti-sense technique was used to suppress 1-aminocyclopropane-1-carboxylate oxidase activity, which catalyses the conversion of 1-aminocyclopropane-1-carboxylate (ACC) to ethylene, had a promotory effect in transgenic *Brassica hirta* (Mustard; Pua and Lee, 1995). A possible mechanism for the effect of ethylene on shoot regeneration is that it inhibits auxin transport (Suttle, 1988). Polar auxin transport is postulated to be essential for the proper establishment of polarity and normal development during embryogenesis (Liu *et al.*, 1993) and the formation of shoot meristems (Cambecedes *et al.*, 1992). It is possible that ethylene induces partial inhibition of polar auxin transport to increase the cytokinin : auxin ratio, facilitating shoot regeneration. Raising the ethylene concentration further may disrupt polarity and normal meristem development.

Ethylene is evolved by *P. avium* shoot cultures (Righetti, *et al.*, 1988), and can be produced at elevated levels when plants are wounded and/or stressed (Perl *et al.*, 1988), so may be present in significant quantities after the excision of leaf explants. The silver ion (Ag^+) is a very

potent inhibitor of ethylene action (Beyer, 1976): the exact mechanism of action has not been elucidated, but is postulated to involve antagonistic interaction with the ethylene binding site resulting in the inactivation of the receptor (Yang, 1985). Conceivably, low adventitious shoot regeneration competence may have resulted from the detrimental effects of ethylene resulting from superfluous tissue damage during wounding. Therefore, the hypothesis that silver nitrate ($AgNO_3$) would promote competence was tested, but no improvement was observed. Escalettes and Dosba (1993) had previously reported that $AgNO_3$ enhanced adventitious shoot regeneration of a number of plum clones from *P. marianna, P. domestica*, and *P. insititia* by up to forty percent, and reduced experiment-to-experiment variation, but their results also suggest this improvement was both species and genotype-dependent. The latter may account for the failure of $AgNO_3$ to improve the shoot regeneration competence of accession 1905. However, $AgNO_3$ treatment did not reduce regeneration competence either, suggesting ethylene is not the principal factor influencing regeneration in this species. Kumar *et al.* (1996) observed that adventitious shoot regeneration from *Paulownia kawakamii* leaf explant was inhibited if aminoethoxyvinylglycine (AVG; an inhibitor of the enzyme catalysing the formation of ethylene precursor ACC) was applied as a medium supplement. However, removal of ethylene from the head space of the culture vessel promoted regeneration. This may result from unknown interaction of AVG *in planta*, or conceivably, there is a requirement for endogenous ethylene in physiological concentrations in the promotion of organogenesis in this species, thus emphasising that the mode of ethylene removal may influence results with other species. If the latter were correct, possibly removal of ethylene from the head space of *P. avium* shoot regeneration cultures would have given a different response.

6. Transformation of *Prunus* with the *Arabidopsis thaliana AUX1* Gene Under *CaMV 35S* Promoter Control

6.1 Introduction

Recombinant DNA technology enables the creation of unique genotypes, promoting the selection of plants with desirable traits. However, the genetic improvement of trees [Tzfira (1998), Walter *et al.* (1998)] lags well behind progress made with many other agricultural commodity crops [Lindsey (1992), James and Krattiger (1996)]. In part, this is because methods germane to the integration of transgene(s), and subsequent regeneration of autonomous plants from transformed cells, remain elusive for many economically important woody species. Nevertheless, the number of transformed species increases apace. Thus far, members of the commercially important *Populus* genus feature foremost amongst genetically transformed tree species, but the number of genotypes where transgenesis is routine remains limited. *Malus* species have also been extensively researched. Stable, *Agrobacterium*-mediated transgenesis has been achieved with several economically important conifer species [e.g., Norway Spruce (*Picea abies*), and European and hybrid Larch (*Larix* spp.); reviewed by Tzfira *et al.* (1998), Walter *et al.* (1998)]. However, for Gymnosperms, transformation efficiency has often been higher with biolistic-based DNA delivery techniques using immature and mature zygotic embryos or isolated megagametophytes as tissues from which somatic embryos are regenerated. This approach continues to expand the number of conifer species transformed [e.g., White and (*Picea glauca*) Black (*Picea mariana*) Spruce, Eastern Larch (*Larix laricina*), Monterey (*Pinus radiata*) and Scots Pines (*Pinus sylvestris*); Ellis *et al.* (1991), Charest *et al.* (1996), Klimaszewska *et al.* (1997), and Walter *et al.* (1998), respectively]. There are many target areas for genetic improvement of trees.

Modification of lignin biosynthesis

Lignin provides mechanical support and defence against pathogens, but impedes chemical-pulping efficiency. However, being relatively energy-rich, it is desirable in wood exploited as an energy source. Thus, manipulation of total lignin content and/or composition is highly desirable. Lignin is heterogeneous, composed of *para*-hydroxyphenyl, guaiacyl and syringyl lignins (Boudet *et al.*, 1995). Syringyl units are easier to hydrolyse than the others, but contain relatively more methoxy groups (CH_3O) which are converted to environmentally polluting mercaptanes during

processing. Transgenic poplar hybrids have been produced with anti-sense *O*-methyltransferase (Van Doorselaere, 1995), or sense and anti-sense cinnamyl alcohol dehydrogenase (Baucher *et al.*, 1996) which catalyses the final step in monolignol biosynthesis. These plants did not have reduced overall lignin content, but did have modified syringyl : guaiacyl ratios, improving extraction efficiency while minimising their propensity to pollute during processing.

Herbicide resistance

Herbicide use is less prevalent in forestry than agricultural environments because weed competition principally affects nursery and young forest plantations before trees outgrow these competitors. However, many tree species succumb to the toxic effects of herbicides before aggressive perennial weeds are killed (Davenhill *et al.*, 1994). Reduced sensitivity to and/or direct detoxification of herbicides has been achieved in a number of transgenic species [e.g., reduced glyphosate sensitivity of hybrid *Populus* (Fillatti *et al.*, 1988) and European larch (Shin *et al.*, 1994), increased phosphinothricin tolerance in hybrid *Populus* (De Block, 1990) and chlorosulfuron resistance (Brasileiro *et al.*, 1992)]. Walter *et al.* (1998) report they are evaluating transgenic *Pinus radiata* plants expressing genes conferring resistance to the less environmentally pernicious herbicides, phosphinothricin and sulfonylurea.

Insect resistance

Insects damage tree growth and form, reducing productivity, but economically viably insecticide use is limited in forestry due to the extensive areas involved, environmental pollution, perceived risk of evolving resistant species and cost. Protection against sap-sucking (Homopetera) insects has been conferred on tobacco plants expressing a snowdrop (*Galanthus nivalis*) lectin -GNA (Hilder *et al.*, 1995). The soil bacterium *Bacillus thuringiensis* (*Bt*) produces parasporal crystals containing endotoxins which are activated in the alkaline digestive system of certain insect larvae (e.g., Lepidopteran species) but non-toxic to humans, many beneficial insects or other wild life. *Bt*-expressing white spruce (Ellis *et al.*, 1993), European larch (Shin *et al.*, 1994), and *Populus* [Kleiner *et al.*, (1995), Wang *et al.* (1996)] had reduced defoliation. However, in caution, the diamondback moth (*Plutella xylostella*), a global pest of vegetable crops, has recently become the first insect known to have evolved resistance to the toxin in open-field populations, raising concern over the evolution of resistance in other pests (Heckel *et al.*, 1999).

Fungal and bacterial resistance

Plant defence resistance genes are highly specific in function. They have been exploited extensively in conventional breeding programmes, but they also offer a significant resource for use in transgenesis. A number of genes expressing anti-bacterial and anti-fungal proteins have been identified. These have potential for conferring cross-species resistance [Everett (1994), Bent *et al.* (1994), Sharma and Looneborg (1996)]. For example, the *A. thaliana RPS2* gene (Bent *et al.*, 1994) confers resistance to *Pseudomonas syringae*, a bacterial pathogen infectious to a number of species including cherry.

Manipulation of reproduction

Reducing the time per generation would be of benefit to tree breeding programmes. The ectopic expression of the flower meristem identity gene *LFY* in hybrid-aspen converted vegetative shoots to reproductive ones, reducing time to flowering from over eight years required for wild-type plants to less than six months (Weigel and Nilsson, 1995). Elis *et al.* (1965) found the growth rate of Douglas fir decreased by approximately 16 percent due to investment in reproduction. Thus, introducing sterility may divert resources into vegetative growth and raise forestry productivity. Within forest environments, potential gene flow between related populations is high (Adams, 1992), thus, sterility would also prevent the dissemination of transgenic material. Among the possible approaches is the expression of cytotoxic genes under promoters specific to reproduction, or the use of antisense constructs for promoter suppression. For example, promoters from homeostatic MADS box genes controlling reproductive development (Strauss *et al.* 1995) are possible candidates.

6.1.1 Transformation of Prunus

Agrobacterium-mediated transformation of *Prunus* spp. has been successful from immature seed and embryo-derived tissues [*P. persica*, Smigocki and Hammerschlag (1991); and *P. domestica*, Mante *et al.* (1991), Scorza *et al.* (1994)]. Notwithstanding the world-wide economic importance of the genus, the sole report of transformation of mature clonal *Prunus* is restricted to an embryogenic line of the cherry rootstock *P. subhirtella autumno rosa* (da Camara Machado *et al.*, 1995). Thus, transformation of mature clonal *Prunus* may be hindered by lack of either effective transformation and/or regeneration procedures.

6.1.2 Prerequisites for transgenesis

Essential components for transgenesis include the availability of appropriate transgene(s). This chapter focuses on the *AtAUX1* gene, which encodes a putative auxin influx carrier. The relevance of this gene is discussed elsewhere (Section *1.4.6*). Additional requirements include, a mechanism for delivering DNA [e.g., direct uptake into protoplasts with or without electroporation, micro-projectile bombardment (including agrolistics; Hansen and Chilton, 1996), or *Agrobacterium*-mediated delivery; Songstad *et al.* (1995, 1996), Gelvin (1998)], a selectable marker [e.g., aminoglycoside antibiotics (Bryan, 1984), isopentenyl-transferase (Ebinuma *et al.*, 1997), or xylose-isomerase (Haldrup *et al.*, 1998)] and a method to regenerate transgenic plants, discussed elsewhere (Section *5*).

6.1.3 Agrobacterium tumefaciens-mediated transgenesis

The genus *Agrobacterium* contains five species: *A. radiobacter, A. rubi, A. vitis, A. rhizogenes* and *A. tumefaciens* (Ophel and Kerr, 1990). Except for *A. radiobacter*, which cannot transform plants, each species transforms specific host plants. *A. tumefaciens* and *A. rhizogenes* are the species most commonly used for plant transgenesis and the former species is the focus of this section.

Transfer DNA (T-DNA)

Wild-type *A. tumefaciens* (the causative agent of crown gall disease) transforms competent plant cells when part of the DNA from a circa 200 kb Ti-plasmid (tumour inducing) is copied, transferred (the T-DNA) and integrates into the host plant genome. Lessl and Lanka (1994) suggest that an evolutionary link exists between this process and bacterial conjugation. The T-DNA is delimited by 25 bp 'border' sequences, of same-orientation, imperfect repeats. The T-DNA of nopaline producing strains is arranged as a contiguous circa 22 kb sequence, whereas, the octopine Ti-plasmid contains three adjacent, independently-transferred, T-DNA sequences. *In planta*, expression of genes encoded within the T-DNA result in production of opines (catabolized by *Agrobacterium* as a source of carbon and nitrogen) and plant growth regulators (i.e., *trans*-zeatin, 2-isopentenyladenine and indole-3-acetic acid) which promote opine output by inducing neoplastic growth. The genes encoding these plant growth regulators are termed oncogenes.

Requirements for Agrobacterium-mediated transformation

Transformation is postulated to involve three elements: the T-DNA complex, plasmid-encoded virulence genes (thus far identified are seven major *vir* loci, *A, B, C, D, E, G* and *H*) and bacterial chromosomal virulence (*chv*) genes [Zambryski (1992), Sheng and Citovsky (1996)]. Additional requirements may include wound-specific inducer molecules (e.g., monocyclic phenolics, and flavonoid or lignin precursors) from plant exudates (Peters and Verma, 1990) and intimate contact between plant cell and *Agrobacterium*. However, Escudero and Hohn (1997) demonstrated that *Agrobacterium* can circumvent the physical barrier of the cuticle via the stomata to transform unwounded tobacco plants. Results of Clough and Bent (1998), with *A. thaliana*, suggest the inclusion of surfactant during cocultivation may enhance *Agrobacterium* uptake into undamaged tissues. Consequently, evoking the wounding response is not always an essential requirement.

Initial contact between phytopathogen and plant cell is secured by *Agrobacterium* produced cellulose fibres (Matthysse, 1996). Products of the *chv* genes (*chvA, chvB, PscA and att*) may be involved in chemotaxis and host cell attachment (Zambryski, 1992). Proteins encoded by the *virH* locus detoxify phenolic compounds that are bacteriostatic at high concentrations. Plant exudates induce the virA/virG two protein component recognition transduction system [directly or possibly via chromosomally encoded proteins, P21 and P10; Winans, *et al.* (1994)]. Induced virA protein receptors (associated with the bacterial inner membrane) autophosphorylate and initiate phosphorylation of the cytoplasmic transducer (virG protein), which in turn activates the *vir* gene complex.

Production and export of T-DNA

Production of the single stranded transferred-DNA (ssT-DNA) complex is postulated to initiate at the 5'RB (right border). Site specific endonucleases (virD1 and virD2) cleave the left and right border sequences, and virD2 forms a high energy phosphotyrosine bond with the 5'RB of the ssT-DNA. A coating of virE2 protein, one per 36 nucleotides (Zambryski, 1992), associates with the ssT-DNA. This association is likely to occur prior to exit from the *Agrobacterium* (Sheng and Citovsy, 1996). Nevertheless, association results in narrowing of the complex to approximately 2 nm, thereby facilitating transport *in planta* and possibly protecting against plant nucleases. After

the protein-nucleic acid complex (T-complex) is excised the Ti-plasmid is restored to a DNA duplex. The T-complex travels towards the plant nucleus with 5'RB/ virD2 leading.

Export of T-DNA into the cytoplasm of the host plant cell is considered to occur via trans-membrane channels constructed with proteins encoded by the *virB* operon. The T-complex must traverse cell walls, several membranes and intracellular spaces before it gains entry to the plant nucleus, via a nuclear pore. Estimates of T-complex size (Howard and Citosky, 1990) suggest active nuclear membrane (NM) influx is necessary. A nuclear localisation signal (NLS) is required for ingress of proteins larger than 40-60 kDa through the NM. Three bipartite NLS (Robbins *et al.*, 1991) are present on the T-complex, one on virD2 and two on the virE2 (Sheng and Citovsky, 1995). The wild-type nopaline T-complex is sixty times the thickness of the NM. Zupan and Zambryski (1995) postulated that the virE2 NLS are involved in keeping both cytoplasmic and nucleoplasmic sides of the nuclear pore open as the T-complex moves through.

Integration of T-DNA into the plant genome

The process of T-DNA integration into the plant genome is uncertain. Models have been proposed for second strand T-DNA synthesis in the nucleus prior to (De Neve *et al.*, 1997) and after (Tinland *et al.*, 1997) integration. Mechanisms requiring a pre-existing gap or nick in one (Gheysen *et al.*, 1991), both (De Neve *et al.*, 1997) or just single strand displacement (Tinland, 1996) of the plant DNA have been postulated. Furthermore, a dichotomy exists as to whether attachment and integration initiates at the 5'RB/virD2 bound end [Zupan and Zambryski (1995), Lessel and Lanka (1994), Zambryski (1995), Gheysen *et al.* (1991), Van Engelen (1995)] or the 3'LB [Tinland (1995, 1996), Sheng and Citovesky (1996), and De Neve *et al.* (1997)] of the T-complex.

Analysis of T-DNA pre-insertion sites (Tinland, 1996) indicates integration is not site-specific, possibly requiring only micro-homology and maybe a preference for transcribed regions. Nevertheless, De Neve *et al.* (1997) found T-DNA from different *Agrobacterium* strains frequently integrated at the same chromosomal locus. Pre-insertion sequences have greater homology with the T-complex 3'LB than the 5'RB. Tinland (1996) proposes integration initiates with plant ssDNA strand displacement and binding of the T-complex 3'LB to a short homologous region. Pairing of a few, perhaps even one, nucleotide occurs at the 5'RB positioning virD2 for its role in ligation.

Displaced plant ssDNA and overhanging 3'LB ssT-DNA is digested. Subsequently, ligation occurs at the 3'LB/5'plant and then 5'RB/3'plant DNA interfaces prior to the plant's DNA repair mechanism(s) copying the second strand. Few nucleotides are lost from the site of insertion, although chromosome rearrangements and inversions have been correlated with T-DNA integration [De Neve *et al.* (1997) and Laufs *et al.* (1999)].

In relation to transforming the plant cell, crucial factors are whether truncation of the T-DNA occurs and at which border. Evidence suggests the T-complex 5'RB is conserved, whereas truncations of up to 100 nucleotides can occur at the 3'LB [Gheysen (1991), Tinland (1996)]. The VirD2 protein maybe involved in ligation of the T-DNA 5'RB/3'plant DNA interfaces (Tinland *et al.*, 1995) suggesting T-DNA 5'RB truncation would separate these components and disrupt insertion. Thus, conservation at the 5'RB is consistent with this model.

T-DNA does not always integrate as single copies - multiple copies (autonomous or associated) in direct or inverted orientation are possible (Gelvin, 1998). Multimers may be biased towards $T\text{-}DNA_{LB}$ involvement in linkage. The presence of repeat T-DNA structures can correlate with transgene silencing.

6.1.4 Plasmid pBIN19

Agrobacterium-mediated plant transformation exploits the naturally occurring capacity for inter-Kingdom exchange of genetic material. Since no enzymes required for transfer and the integration of T-DNA are encoded within the transferred region, foreign genes can substitute for most of the wild-type sequence without compromising transformation efficiency [Zambryski (1992), Zupan and Zambryski (1995)].

The popular and versatile plasmid pBIN19 (Bevan, 1984) was among several pioneering vectors developing the concept of the binary vector transformation system [Hoekema, *et al.* (1983), De Framond, *et al.* (1983)]. The 'classic' system comprises a disarmed Ti-plasmid providing the virulence and transfer functions in *trans*, with a second engineered 'binary' plasmid upon which the T-DNA and replication functions reside. Binary vectors have several advantages over co-integrative vectors based on wild-type Ti-plasmids: they are smaller making them easier to manipulate and contain a broad host-range origin of replication (*oriV*) allowing replication within *E.coli* and *Agrobacterium* (Schmidhauser, 1985). Plasmid pBIN19 has fifteen unique

restriction sites located within a multiple cloning site (MCS) derived from M13mp19 (Norrander *et al.*, 1983), which is positioned inside a *LacZ* gene to provide a convenient blue/white screening of recombinants in *E.coli*; it contains the pRK2 origin of replication. Furthermore, at 11777 bp., pBIN19 has been fully sequenced (Frisch, 1995) aiding location of restriction sites and the precise size prediction of restriction fragment. Selection of putative transformants can be achieved with several aminoglycoside antibiotics, (e.g., kanamycin mono-sulphate, neomycin or G418). *Neomycin phosphotransferase* (*NPT*) II and III inactivate aminoglycoside antibiotics by catalysing the transfer of the terminal phosphate of ATP to the antibiotic (Bryan, 1984). The *NPTIII* gene of pBIN19 is active in *Agrobacterium* and *E.coli* whereas *NPTII* is located within the T-DNA and functions *in planta*.

Potential disadvantages of pBIN19

Yenofsky *et al.* (1990) have identified a mutation within the *NPTII* gene of various stocks of this vector and several derivative constructs. The mutation results from a G to T substitution at nucleotide position 2096, producing a glutamic to aspartic acid conversion at position 182 of the amino acid sequence of the protein. This occurs within a highly conserved region of the enzyme near the carboxyl-terminus believed to be the active site for binding ATP (Bryan, 1984). Evidence suggests the mutation reduces transformation efficiency: recovery of transgenic *Brassica napus* shoots doubled when the mutant *NPTII* gene was replaced with a wild-type copy (Datla *et al.*, 1992), and relative to cultures transformed with the wild-type gene, the fresh masses of mutant *NPTII* transgenic *N. tabacum* cultures maintained on the aminoglycoside antibiotic G418 were fifty percent lower (Yenofsky *et al.*, 1990).

The position of *NPTII* in pBIN19 may be problematic. The gene is located upstream of the MCS near the 5'RB of the T-DNA, which may increase the risk of transformants carrying the marker gene but not the gene of interest. The potential for T-DNA truncation has influenced vector design [McBride and Summerfelt (1990), Van Engelen (1995)].

Superfluous sequences are present within the T-DNA (Fray *et al.*, 1994) and the non T-DNA (Frisch *et al.*, 1995) regions of pBIN19. Frisch *et al.* (1995) concluded a reduction of more than fifty percent would be feasible if non-functional and non-essential regions were removed.

Low plasmid copy number and instability are difficulties with pBIN19. The broad host-range origin of replication, *oriV* from pRK2, provides this plasmid with a useful asset, but this replicon can be unstable [McBride and Summerfelt (1990), Schmidhauser (1985)] and reportedly contributes to the relatively low plasmid copy number (Van Englen, 1995).

Improved pBIN19 derivatives

The pRD400 plasmid series (Datla, 1992) were constructed to rectify the mutant *NPTII* gene. Consequently, apart from the correction to the point mutation the plasmid is exactly as pBIN19. However, Van Engelen (1995) produced plasmid pBINplus, containing wild-type *NPTII* located downstream of the MCS and an ColE1 origin of replication in addition to the functional pRK2 origin. Nevertheless, both retain extraneous fragments and because of size are sub-optimal for efficient manipulation.

6.1.5 Chapter aims

Having improved adventitious shoot regeneration from *P. avium* leaves (Chapter *5*) over that previously reported by Hammatt and Grant (1998), this chapter reports on the attempt to transform *P. avium* and *P. padus* with a cDNA copy of the *AtAUX1* gene, under the control of a cauliflower mosaic virus *35S* promoter (*35S::AtAUX1*). Several plasmid constructs, with or without *35S::AtAUX1*, containing either wild-type or mutant *NPTII*, were constructed to investigate whether this defect affected transformation efficiency. Tobacco and *Arabidopsis* transgenesis was used to further investigate why constructs containing wild-type *NPTII* without *35S::AtAUX1* were unable to transform *P. avium* and *P. padus*, and provided the control material necessary to support conclusions drawn from *35S::AtAUX1* transgenic *P. padus.*

6.2 Materials and Methods

6.2.1 Plant materials

Prunus

Prunus avium and *P. padus* shoot cultures were initiated and maintained as described previously (Chapter *2*) and provided a source of leaf explants. *P. avium* leaves were excised from these cultures as described elsewhere (Section *5.2.1*). *P. padus* leaf explants were prepared in a similar way, except fully expanded unfurled leaves (5-10 mm long) were used.

Arabidopsis thaliana

Wild-type *A. thaliana*, ecotype Wassilewskija (WS), seeds were obtained from Prof. M. J. Bennett (University of Nottingham, Nottingham, UK). Seeds were surface-sterilised [30 s., 76 % (v/v) ethanol, followed by 10 min. in 20 % (v/v) aqueous solution of commercial bleach solution 'Domestos' (Lever Bros., UK), containing not less than 0.5 % (w/v) final NaOCl concentration] and rinsed with sterile water 4-6 times. Following surface-sterilisation, seeds were air dried under aseptic conditions on sterile filter paper and stored in 9 cm Petri Dishes (4 °C, darkness, under desiccation).

Seeds were germinated, *in vitro*, on ***Ar****abidopsis* seed germination medium 1 (ARM1), which consisted of growth-regulator-free, semi-solid, modified MS-medium (Murashige and Skoog, 1962) supplemented with 29.3 mM sucrose (20-30 seeds per SPD). Seven days after germination, *Arabidopsis* seedlings were transferred to a 3 : 1 (v/v) sterilised peat-based compost (type C2; Levington, UK) and sand mixture. Plants were maintained with short day illumination [8 h photoperiod of 10-20 μmol m^{-2} s^{-1} irradiance (PAR) from 8 W type 33 fluorescent tubes, at 25 ± 2 °C] for 14 d to produce a compact rosette, then transferred to a 16 h photoperiod to promote floral development and bolting.

Nicotiana tabacum

Seed of the commercially available tobacco cv. 'Maryland Mammoth' was obtained via Dr. S. Jackson (Horticulture Research International, Warwick, CV35 9EF, UK) from stocks maintained by the Institut fur Pflanzenbau und Pflanzenzuchtung Bundesforschungsanstalt fur Landwirtchaft Braunschweig-Volkenrode (FAL), Bundesallee 50, D-3300 Braunschweig, Germany.

Seeds were surface-sterilised, as described previously for *Arabidopsis*, and transferred to tobacco seed germination and node culture medium 1 (TOM1), which consisted of growth-regulator-free, semi-solid, modified MS-medium (Murashige and Skoog, 1962) supplemented with 58.5 mM sucrose.

A culture line was established on 10th November 1998 from an arbitrarily selected seedling, and propagated by transferring stem node cuttings of approximately 2 cm in length, singly to TOM in HJs, at 28 d intervals.

When required, leaf discs (4 mm dia.) were excised from tobacco cultures at the end of a subculture period, avoiding the mid-rib, while leaves were bathed in liquid TOM1 to prevent desiccation. Explants were arranged with their abaxial surface in contact with the medium.

6.2.2 Transformation/regeneration media

Prunus

P. avium leaf explants were maintained on REM1, as described previously (Section *5.2.2*), but this was supplemented with 10 mgl^{-1} Tween-20. For *P. padus* cultures, basic regeneration medium 2 (REM2) consisted of growth-regulator-free, semi-solid, Woody Plant Medium (WPM; Lloyd and McCown, 1981) supplemented with 0.54 µM 1-naphthaleneacetic acid (NAA; Sigma, Dorset, UK), and 4.4 µM benzyladenine (BA; Sigma, Dorset, UK).

Nicotiana

Basic tobacco regeneration medium 3 (REM3) consisted of growth-regulator-free, semi-solid, modified MS-medium (Murashige and Skoog, 1962) supplemented with 60.5 µM glucose (Duchefa, Haarlem, NL), 4.4 µM BA, and 1.08 µM NAA.

Unless stated otherwise, agar concentration, pH adjustment, autoclaving, media dispensing volumes, and culturing conditions were as described elsewhere (Section *2.2*).

6.2.3 Agrobacterium strains and plasmids

A 1.9 kb cDNA copy of the *AtAUX1* gene, ligated into pROK2 (designated pROK2*AUX1*) as a *BamHI*/*Eco*RI fragment under cauliflower mosaic virus (*CaMV*) *35S* promoter and terminator control, was kindly provided by Prof. M. J. Bennett. To identify pBIN19 derivative plasmids that carried wild-type or mutated *NPTII* genes, *Xho*II restriction digests were carried out (in

accordance with the methods of Yenofsky *et al.*, 1990) on pROK2*AUX1*, pBIN19 (Bevan, 1984), pRD400 and pRD410 (Datla *et al.*, 1990) and resolved through 1.5 % (w/v) agarose gel electrophoresis containing 1.27 mM ethidium bromide. In mutant plasmids, the loss of a restriction site results in a 638 bp restriction fragment instead of the 246 bp and 392 bp fragments produced from a wild-type gene (Yenofsky *et al.*, 1990).

*Bam*HI/*Eco*RI restriction digests of pROK2*AUX1* and pRD410 released *AtAUX1*::*CaMV35S* $_{terminator}$ and *UIDA*::*NOS* $_{terminator}$ fragments from each plasmid, respectively. The *AtAUX1*::*CaMV35S* $_{terminator}$ and pRD410 (retaining a *CaMV35S* promoter) fragments were purified by 1.5 % (w/v) agarose gel electrophoresis, recovered [30 min. -20 °C, centrifuged 5 min. 13000 rpm. through a 'spinex' filtration column, in accordance with the manufacturers instructions (Costar Ltd, UK)], ligated together to produce plasmid pRD400*AUX1*, and electroporated into *E. coli* strain DH5α, using procedures described in Sambrook *et al.* (1989).

Plasmids pRD400*AUX1* (wild-type *NPTII* with *35S::AtAUX1*), pROK2*AUX1* (mutant *NPTII* with *35S::AtAUX1*), pRD400 (wild-type *NPTII* without *35S::AtAUX1*) and pBIN19 (mutant *NPTII* without *35S::AtAUX1*) were electroporated into *A. tumefaciens* strain C58pMP90 (Koncz and Schell, 1986) using the method described by Wen-jun and Forde (1989).

6.2.4 Inoculation, cocultivation, and 'pseudo-cocultivation'

Agrobacterial suspensions

Agrobacteria stocks were maintained at – 80 °C in YEP medium [85.56 mM NaCl (Merck, UK), 1 % (w/v) yeast extract and tryptone (Oxoid, UK), pH 7] supplemented with 15 % (v/v) glycerol (Merck, UK) and 50 mg l^{-1} kanamycin mono-sulphate and provided inoculum for establishing overnight suspension cultures, in medium of similar composition but without glycerol [10 ml medium per 30 ml universal bottle of polystyrene body and polyethylene cap construction (Greiner Labortechnik, UK), 120 rpm., 28 °C, darkness]. This culture provided a 1:20 (v/v) inoculum for establishing an overnight suspension culture, which was centrifuged (5 min., 2500 rpm.) and resuspended in kanamycin-mono-sulphate-free YEP medium (10 ml) for the cocultivation of explants.

Cocultivation of Prunus and Nicotiana leaf explants

Excised leaf explants were inoculated by immersion into an agrobacterial suspension (approximately 20 s., 10 ml of suspension within a 9 cm PD), and excess inoculum was shed onto the inner surface of the PD before they were transferred to the appropriate medium (20 leaves and 25 ml medium per dish) for a 72 h period of cocultivation. Following cocultivation, leaves were transferred to medium of similar composition supplemented with 400 mg l^{-1} cefotaxime sodium (CTX; Roussell, Uxbridge, UK) to eliminate agrobacteria, and kanamycin mono-sulphate (KM; Sigma, Dorset, UK), at various concentrations, depending on individual experiments, to select transgenic callus and shoots.

Eradication of agrobacteria was achieved by maintaining a CTX concentration of 400 mg l^{-1} for two subcultures followed by two further subcultures with CTX at 200 mg l^{-1}, with a 28 d interval between successive subcultures. Both antibiotics were filter-sterilised through 0.2 μm syringe filters (Whatman, Kent, UK) and added to post-autoclaved medium that had been cooled to between 40-50 °C.

'Pseudo-cocultivation'

Control explants were subjected to a 72 h 'pseudo-cocultivation' where they were inoculated with medium filtrate from replicate suspensions to those used during cocultivations with *Agrobacterium*. This filtrate was prepared by filtering a fresh agrobacterial suspension through a 0.2 μm syringe filter twice. To ensure the filtrate was free from agrobacteria, it was incubated for 48 h after carrying out inoculation at identical conditions used to produce the suspension cultures.

Cocultivation of Arabidopsis inflorescences

A. thaliana plants and Agrobacterial suspensions were prepared as described previously. However, these suspensions were supplemented with 0.03 % (v/v) 'Silwet L 77' surfactant (Lehle Seeds, USA) prior to carrying out cocultivations.

Plants were either pseudo- or *Agrobacterium*-cocultivated, when inflorescences had developed to 4-8 cm in length, by pipetting Agrobacterial suspension, or filtrate, (approximately 100 μl per plant) onto unopened floral buds (2 applications 4 d apart). Plants were segregated

from neighbours by their enclosure in open-ended plastic tubes, which were sealed for 3 d after each application to promote humidity and prevent desiccation.

6.2.5 Experiment 6.1: Determining the kanamycin mono-sulphate concentration required to inhibit adventitious shoot regeneration from wild-type P. avium leaves

Per treatment, 25 leaf explants from accession 1908 and/or 1905 were distributed equally amongst five 9 cm PDs containing REM1 supplemented with KM at the following concentrations:

- ***a:*** accession 1908 with 0, 5, 10, 15, 20 or 25 mg l^{-1}.
- ***b:*** accession 1908 with 0, 15, 25, 35, 45 or 55 mg l^{-1}
- ***c:*** accession 1908 with 0, 15, 25, 35, 45 or 55 mg l^{-1}, and 1905 with 0, 25, 35 and 45 mgl^{-1}
- ***d:*** accession 1905 with 0, 35, 45, 55, 65 or 75 mg l^{-1}.

Data were recorded 28 d after excision of leaves. Individual experiments were carried out between May and July 1997, and non-replicated.

6.2.6 Experiment 6.2: Attempt to transform P. avium with 35S::AtAUX1

Leaf explants from accession 1905 were cocultivated on REM1 (100 leaves per 14 cm PD, 125 ml medium) with *Agrobacterium* constructs containing wild-type *NPTII*, with and without *35S::AtAUX1*, or pseudo-cocultivated.

Following cocultivation, explants were transferred to REM1 supplemented with CTX and KM in the following proportions:

- explants cocultivated with *Agrobacterium* with *35S::AtAUX1*, 475 leaves (evenly distributed in 14 cm PDs) on 55 mg l^{-1}, and 25 leaves (5 per 9 cm PD) each on 0, 65 and 75 mg l^{-1} KM.
- explants cocultivated with *Agrobacterium* without *35S::AtAUX1*, 475 leaves (evenly distributed in 14 cm PDs) on 55 mg l^{-1}, and 25 leaves (5 per 9 cm PD) on medium without KM.
- pseudo-cocultivated explants, 25 each (5 per 9 cm PD) on medium without and with 55 mg l^{-1} KM.

After a further 28 d of culture, explants were subcultured to fresh media of the same composition, and data were collected 28 d later. This experiment was not replicated, and carried out during July and August 1997.

6.2.7 *Experiment 6.3: Determining the kanamycin mono-sulphate concentration required to inhibit adventitious shoot regeneration from wild-type P. padus leaves*

Per treatment, 24 leaf explants were distributed equally amongst five 9 cm PDs containing REM2 supplemented with either 0, 5, 10, 15, 20 or 25 mg l^{-1} KM. Data were collected after 28 d of culture, and explants were subcultured onto fresh media of similar composition, and recorded 28 d later. This experiment was carried out between February and April 1997.

6.2.8 *Experiment 6.4: Transformation of P. padus with 35S::AtAUX1*

Experiment a: Leaf explants were cocultivated on REM2 with *Agrobacterium* constructs containing either wild-type or mutant *NPTII*, with or without *35S::AtAUX1*, or were pseudo-cocultivated (20 leaves each per 9 cm PD).

Following cocultivation, explants were transferred individually to CCPs of REM2 supplemented with CTX and either 0, 25, 50 or 75 mg l^{-1} KM, giving 20 treatments of 20 leaves each. Twenty-eight days later, explants were subcultured to fresh medium of similar composition, and thereafter subcultured at 21-28 d intervals for circa 8 months. Initial data were collected 56 d after cocultivation. KM and CTX supplementation of medium ceased 112 d after cocultivation.

Experiment b: Leaf explants were cocultivated on REM2 with *Agrobacterium* constructs containing wild-type *NPTII*, with or without *35S::AtAUX1*, or pseudo-cocultivated (20 leaves per 9 cm PD).

Following cocultivation, explants were transferred individually to CCPs of REM2 supplemented with CTX and either 0 or 35 mg l^{-1} KM (8 treatments in total with 20 leaves each). Cultures were maintained as in experiment *a* until 56 d post-cocultivation when 24 independently derived putative transgenic calluses were excised from the remainder of the explant and subcultured every 7 d to fresh REM2 with 400 mg l^{-1} CTX and 35 mg l^{-1} KM.

Experiment c: Leaf explants were cocultivated on REM2 with *Agrobacterium* constructs containing wild-type *NPTII* and *35S::AtAUX1* (200 explants with 100 per 14 cm PD), or pseudo-cocultivated (40 explants with 20 per 9 cm PD).

Following cocultivation, *Agrobacterium*-cocultivated explants were transferred individually to CCPs of REM2 supplemented with CTX and 25 mg l^{-1} KM; and pseudo-cocultivated explants were transferred separately to CCPs of REM2 supplemented with CTX and either 0 or 25 mg l^{-1} KM (20 per treatment).

Subsequent treatment of cultures was identical to that in experiment *a*, until 56 d post-cocultivation when *Agrobacterium*-cocultivated explants were divided into two groups. Twenty-four independently derived calluses were maintained on REM2 with CTX and 35 mg l^{-1} KM. The remaining calluses (344) were transferred to REM2 supplemented with CTX, but without KM. Both groups of cultures were subcultured at 21-28 d intervals.

As they arose, putative (kanamycin mono-sulphate resistant) transgenic shoots were transferred to SHM3 and multiplied for further analysis and experimentation. Experiments *a* and *b* were carried out primarily during 1997, with experiment *c* during 1998.

6.2.9 Experiment 6.5: Determining the kanamycin mono-sulphate concentration required to inhibit adventitious shoot regeneration from wild-type tobacco leaf discs

Leaf discs (20 per treatment) were excised and transferred singly to CCPs containing REM3 supplemented with 0, 50, 75, 100 or 125 mg l^{-1} KM. Explants were subcultured to fresh medium of similar composition after 28 d of culture. Data were recorded after 28 and 56 d of culture, during December and January 1998/99.

6.2.10 Experiment 6.6: Transformation of tobacco with 35S::AtAUX1

Experiment *a*: Leaf discs were cocultivated on REM3 with *Agrobacterium* constructs containing wild-type *NPTII*, with or without *35S::AtAUX1*, or pseudo-cocultivated (48 discs per treatment distributed equally between two 9 cm PD).

Following cocultivation, discs were transferred singly to CCPs containing REM3 supplemented with CTX, with and without 50 mg l^{-1} KM (24 discs per treatment). Leaf discs were subcultured to fresh medium of similar composition after 28 d and 56 d of culture. Initial data were recorded after 56 d of culture (March 1999).

Twenty cultures, with putative transgenic shoots, were selected arbitrarily across both replicate experiments (10 each) from those cocultivated with *Agrobacterium* containing wild-type *NPTII* and *35S::AtAUX1*. Five shoots were excised from each culture and transferred singly to

CCPs containing TOM1 supplemented with CTX and either 50, 75, 100, 125, or 150 mg l^{-1} KM, and subcultured onto fresh medium of similar composition after 28 d of culture. Following a further 28 d of culture, surviving shoots were transferred to TOM1 supplemented with 150 mg l^{-1} KM, and subcultured to fresh medium of a similar composition twice more, at 28 d intervals.

***Experiment b*:** Leaf discs were cocultivated on REM3 with an *Agrobacterium* construct containing wild-type *NPTII* and *35S::AtAUX1*, or pseudo-cocultivated. (40 discs per treatment distributed equally in two 9 cm PD).

Following cocultivation, discs from each treatment were transferred singly to CCPs containing REM3 supplemented with CTX, with and without 150 mg l^{-1} KM (20 discs each), and subcultured to medium of a similar composition after 28 d of culture. Subsequently, a single shoot was excised from each of twenty arbitrarily selected *Agrobacterium* and pseudo-cocultivated cultures, previously maintained either with or without KM, respectively. These were transferred to TOM1 supplemented with CTX and 150 mg l^{-1} KM, and subcultured to fresh medium of similar composition after 28 d of culture.

6.2.11 Experiment 6.7: Transformation of Arabidopsis with 35S::AtAUX1

Per treatment, the inflorescences of three *A. thaliana* plants (designated T_0 plants), were cocultivated (Section *6.3.4*) with *Agrobacterium* carrying wild-type *NPTII*, with or without *35S::AtAUX1*, or pseudo-cocultivated.

Siliques matured within the next 14 d, after which time plants were allowed to dry out naturally and seed (T_1 seed) was harvested 21 d later. Inflorescences, with brown dry siliques, were gently rubbed between thumb and forefinger over clean paper to release seed, which was sieved 4-6 times to remove the bulk of any contaminating waste plant material.

Per treatment, 750–1000 seeds (T_1 seed from self-fertilised T_0 plants) were surface-sterilised and distributed equally between 5 SPDs, containing growth-regulator-free, semi-solid, modified MS-medium (Murashige and Skoog, 1962; Imperial Laboratories, Dorset, UK) and 29.23 mM sucrose supplemented with 400 mg l^{-1} CTX, with or without 35 mg l^{-1} KM.

After 10 d of culture, 20 arbitrarily selected putative transgenic seedlings (T_1 seedlings), which appeared resistance to bleaching in the presence of KM, were transferred singly to pots

with 3 : 1 (v/v) sterilised peat-based compost (type C2; Levington, UK) sand mixture, and plants were self-fertilised to produce T_2 seed.

T_2 seed (from each of the 20 selected T_1 plants) was harvested, surface-sterilised and subjected a further round of selection, under similar conditions to that used with T_1 seed, except the concentration of KM was increase to 50 mg l^{-1}. The segregation pattern of T_2 seed was derived from resistance to bleaching seen in the germinated T_2 seedlings.

From each T_2 seedling population, expressing a desirable segregation ratio, 10 resistant seedlings were selected arbitrarily. These were transferred singly to compost and self-fertilised to produce T_3 seed, which was harvested, surface-sterilised and subjected a further round of selection. The resulting T_3 seedlings were scored for resistance to bleaching.

6.2.12 Polymerase chain reaction (PCR) analysis of putative 35S::AtAUX1 transgenic cultures

Rapid DNA extraction method

Putative transgenic tissue and *Agrobacterium* samples were flash frozen in liquid nitrogen (in 1.5 ml vol. eppendorf tubes) and ground with aluminium oxide powder (approximately 40 µl vol. per tube) and a disposable pestle encrusted with aluminium oxide (created by dipping the heated molten end of a 1000 µl pipette tip into Al_2O_3 powder). Grinding continued (10 min., 65 °C) with the addition of 500 µl of modified Doyle and Doyle (1990) extraction buffer [1.4 M sodium chloride, 20 mM ethylenediaminetetra acetic acid (EDTA), 100mM Tris-HCl (pH 8), 4 % (w/v) cetyltrimethylammonium bromide (CTAB), 2 % (w/v) polyvinylpyrrolidone (PVP-40), and 1 % (v/v) mercaptoethanol]. Nucleic acids were separated from proteins with dichloromethane : isoamyl alcohol [24:1 (v/v); 500 µl] vortexing and centrifugation (10 min., 13000 rpm.), and precipitated from the transferred supernatant with isopropanol (500µl), gentle inversion and centrifugation (5 min., 13000 rpm.). The supernatant was discarded, and an equal volume of wash buffer added [76 % (v/v) ethanol and 10 mM ammonium acetate] followed by gentle inversion and centrifugation (5 min., 13000 rpm.). Nucleic acids were air dried, and the pellet resuspended in 50 µl of sterile water by incubation for 15 min. at 37 °C (all chemicals from Sigma, Dorset, UK).

Conditions of the polymerase chain reaction (PCR)

PCR reactions (50 µl final volume) were carried out in buffer consisting of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNPT, 1.25 U *Taq* polymerase and 0.5 µM of each primer (all obtained from Life Technologies, UK) and 5 µl of each DNA sample. The PCR heating block conditions were denature at 94 °C for 30 s and 35 cycles at 94 °C for 30 s, 55 °C for 120 s, 72 °C for 180 s with a final step of 72 °C for 420 s. PCR products were separated on 1.2 % (w/v) agarose gels containing 0.5 µg ml^{-1} ethidium bromide and visualised under UV light.

Primers for the *Agrobacterium VirD1* gene [positions 1827-1849 and 2242-2264, with an expected 450 bp product; Hirayama *et al.* (1988)] were used to confirm eradication of Agrobacteria from cocultivated plant tissues. Confirmation of putative *35S::AtAUX1* transformants was achieved with primers for within the CaMV 35S promoter (5'-CAC GCT GGG GGA TCC CCA CTC-3') and the *AtAUX1* gene, for an expected 510 bp product (5'-AAA TGC GAG ACC TAG TGC-3') or for a longer, virtually complete span of the transgene, (5'-AAC ACT TGG CAA AGA GAC-3') giving an expected product of 1463 bp

DNA extraction and PCR analyses were replicated at least twice for each putative transgenic line.

6.2.13 *Experiment 6.8: Assessing the capacity of 35S::AtAUX1 transgenic P. padus leaves to produce adventitious shoots*

Per treatment, 25 leaf explants from wild-type and six *35S::AtAUX1* transgenic shoot culture lines were distributed equally amongst 5 PDs (9 cm) containing REM2 with or without 25 mg l^{-1} KM. Following 28 d of culture, these were transferred to fresh medium of the same composition, and data were recorded 28 d later. This experiment was carried out between January and March 1999)

6.2.14 *Experimental designs*

Unless stated otherwise, all experiments were randomised in blocks, containing one PD or CCP of each treatment, and repeated twice.

6.2.15 *Statistical analyses*

Where statistical analyses were carried out, this was achieved using Genstat V software (Genstat 5 Committee, 1993). The normal approximation to the binomial (*z*) was used for

comparing the proportions of leaves that regenerated shoots in experiment 6.3. In experiment 6.8, generalised linear models (McCullagh and Nelder, 1989) with binomial error and the logit link function were used to compare the proportions of leaves with shoots. Mass of callus was subjected to log transformation and analysed by ANOVA. The threshold for statistical significance was taken to be the P=0.05 probability level.

6.3 Results

6.3.1 *Experiment 6.1: The concentration of kanamycin mono-sulphate required to inhibit adventitious shoot regeneration from wild-type P. avium leaves*

Experiment *a*: 44 percent of leaf explants produced at least one adventitious shoot on REM1 supplemented with 25 mg l^{-1} KM, the highest concentration assessed (Table *6a*), which was not significantly different from regeneration on kanamycin-free-medium suggesting a higher concentration would be needed to inhibit regeneration.

***Experiments b, c and d*:** relative to cultures maintained on kanamycin-free-medium, 25 mg l^{-1} KM significantly reduced the proportion of leaf explants with adventitious shoots (Table *6a*). No cultures developed adventitious shoots on a medium supplemented with 55 mg l^{-1} KM or greater. However, data suggest a degree of experiment-to-experiment variation, and there is a high probability of error associated with regeneration frequencies of zero and one for these relatively small sized samples. Nevertheless, 55 mg l^{-1} KM was chosen as the lowest concentration required for selection in the subsequent transformation experiment.

Table 6a. Effect of kanamycin mono-sulphate concentration on the number of wild-type *P. avium* leaves producing adventitious shoots

Kanamycin mono-sulphate concentration (mg l^{-1})	Accession	1908		1905	
	Experiment				
	a	*b*	*c*	*c*	*d*
0	12	15	18	21	19
5	14	-	-	-	-
10	19	-	-	-	-
15	11	9	10	-	-
20	8	-	-	-	-
25	13	6	2	3	4
35	-	0	2	8	1
45	-	0	0	2	0
55	-	0	0	-	0
65	-	-	-	-	0
75	-	-	-	-	0

- Concentration was not assessed in this experiment
Values indicate the number of leaves with at least one shoot, based on 25 replicates per treatment
Data were recorded 28 d after excision of leaves

6.3.2 *Experiment 6.2: Cocultivation of P. avium leaves with Agrobacterium constructs with wild-type NPTII and 35S::AtAUX1*

An attempt to transform *P. avium* with *35S::AtAUX1* was made. On kanamycin-sulphate-free REM1, there was no difference between treatments (pooled proportion = 56 %; Table *6b*). On KM-supplemented medium, no pseudo-cocultivated explants regenerated, and from 1000 *Agrobacterium*-cocultivated explants, only two shoots were produced. Both shoots were small and malformed and developed on medium supplemented with 55 mg l^{-1} KM, the lowest concentration assessed. On transfer to fresh medium of the same composition these shoots rapidly necrosed. These results suggested further development would be required to achieve transformation of *P. avium*.

Table 6b. Number of *P. avium* (accession 1905) leaves producing shoots on medium supplemented with kanamycin mono-sulphate after cocultivation with *Agrobacterium* C58pMP90 constructs containing pBIN19 derivatives with wild-type *NPTII*, with or without *35S::AtAUX1*

Leaves inoculated with	Kanamycin mono-sulphate concentration (mg l^{-1})				
	0	55	65	75	Total
+35S::AtAUX1	16	1[a]	0	0	17
- 35S::AtAUX1	11	1[a]	-	-	12
Pseudo-cocultivated	15	0	-	-	15
Total	42	2	0	0	

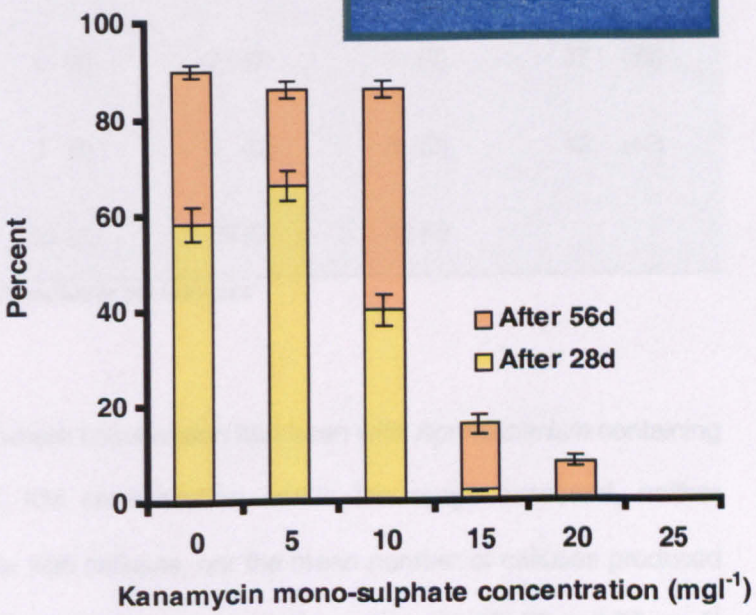
[a] Values indicate the number of leaves with at least one shoot, based on 475 replicates per treatment, whereas, all other treatments contained 25 leaves
- Indicates that this treatment was not assessed in this experiment
Data were recorded 56d after cocultivation

6.3.3 *Experiment 6.3: The concentration of kanamycin mono-sulphate required to inhibit adventitious shoot regeneration from wild-type P. padus leaves*

As *P. avium* appeared difficult to transform with *35S::AtAUX1*, *P. padus* was evaluated as an alternative.

The proportions of wild-type leaves with adventitious shoots, after 56 d of culture, were not significantly different (P=0.05) on medium without, or with either 5 or 10 mg l^{-1} KM (Figure *6a*). The proportions of leaves with shoots increased between 28 and 56 d of culture (P<0.01). The capacity of leaves to regenerate shoots was reduced significantly on REM2 with 15 or 20 mg l^{-1} KM, and was completely inhibited at 25 mg l^{-1} KM. In contrast, 90 percent of leaves on medium without KM regenerated at least one shoot. Consequently, a concentration of 25 mg l^{-1} KM was chosen for the selection of transgenic shoots in subsequent experiments with *P. padus*.

Figure 6a. The effect of supplementing REM2 with kanamycin mono-sulphate on the proportions of wild-type *P. padus* leaves producing adventitious shoots (insert: a regenerating explant; scale bar = 10 mm)



Values were derived from two experiments of 24 replicates per treatment (±SE)

6.3.4 *Experiments 6.4 a, b and c: Effect(s) of the 35S::AtAUX1, and wild-type or mutated forms of the NPTII gene on the transformation efficiency of P. padus leaves*

These experiments had the aims of transforming *P. padus* with *35S::AtAUX1* and assessing the hypothesis that a mutation within *NPTII* may have reduced transformation efficiency.

In experiments *a* and *b* (Tables *6c-d*), on medium supplemented with KM, significantly greater proportions of explants produced calluses where cocultivation had been with

Agrobacterium containing wild-type *NPTII* and *35S::AtAUX1* (pooled proportion = 79 %). Similarly, in experiment *c* (Table *6e*), 92 percent of explants cocultivated with this construct produced calluses. In contrast, where cocultivation occurred with any one of the other constructs, callus formation was almost completely inhibited in the presence of KM. This suggests that the mutation within *NPTII* is unlikely to be directly responsible.

Table 6c. Number of *P. padus* leaves producing callus and shoots (in parentheses) on medium supplemented with kanamycin mono-sulphate after cocultivation with *Agrobacterium* C58pMP90 carrying pBIN19 derivatives containing wild-type or mutant *NPTII*, with or without *35S::AtAUX1*

Leaves inoculated with	Kanamycin mono-sulphate concentration (mg l^{-1})				
	0	25	50	75	Total
Wild-type *NPTII* - *35S::AtAUX1*	39 (39)	1 (0)	0 (0)	0 (0)	40 (39)
+ *35S::AtAUX1*	39 (36)	25 (1)	38 (0)	32 (0)	134 (37)
Mutant *NPTII* - *35S::AtAUX1*	37 (37)	0 (0)	0 (0)	0 (0)	37 (37)
+ *35S::AtAUX1*	36 (36)	1 (0)	0 (0)	1 (0)	37 (36)
Pseudo-cocultivated	40 (40)	2 (2)	0 (0)	0 (0)	42 (42)
Total	191 (188)	29 (3)	38 (0)	33 (0)	

Values were derived from two experiments of 20 replicates per treatment
Data were recorded 56d after cocultivation

In experiment *a* (Table *6c*), where cocultivation had been with *Agrobacterium* containing wild-type *NPTII* and *35S::AtAUX1*, KM concentration, within the range assessed, neither influenced the proportions of explants with calluses, nor the mean number of calluses produced per explant (i.e., 1.6, 2.4 and 2.08 calluses, on medium supplemented with 25, 50 and 75 mg l^{-1} KM, respectively). This supports the view that the mutation within *NPTII* does not directly reduce the formation of putatively transformed *P. padus* calluses.

Across all three experiments (Tables *6c-e*), the proportions of pseudo- or *Agrobacterium*-cocultivated leaves which regenerated adventitious shoots on kanamycin-sulphate-free medium did not differ significantly (pooled proportion = 97 %). However, shoot regeneration was reduced under selective conditions. On medium with 25 mg l^{-1} (experiment *a*, Table *6c*) or 35 mg l^{-1} KM (experiment *b*, Table *6d*) low proportions of pseudo-cocultivated

explants produced calluses (3.75 %) and shoots (2.5 %), but in experiment *c*, none formed on medium with 25 mg l^{-1} KM (Table *6e*), nor in experiment *a* (Table *6c*) with a concentration of 50 mg l^{-1} KM or greater. Thus, 25-35 mg l^{-1} KM was sufficient to prevent all but a few non-transformed shoots from regenerating.

Across all experiments, from a total of 494 explants cocultivated with *Agrobacterium* containing wild-type *NPTII* and *35S::AtAUX1* and maintained on medium with KM, only three produced single adventitious shoots (pooled proportion = 0.6 %).

Table 6d. Number of *P. padus* leaves producing callus and shoots (in parentheses) on medium supplemented with kanamycin mono-sulphate after cocultivation with *Agrobacterium* C58pMP90 carrying pBIN19 derivatives containing wild-type *NPTII*, with or without *35S::AtAUX1*

Leaves inoculated with	Kanamycin mono-sulphate concentration (mg l^{-1})		
	0	35	Total
- *35S::AtAUX1*	38 (38)	0 (0)	38 (38)
+ *35S::AtAUX1*	40 (40)	31 (1)	71 (41)
Pseudo-cocultivated	39 (39)	1 (0)	40 (39)
Total	117 (117)	40 (1)	

Values were derived from two experiments of 20 replicates per treatment
Data were recorded 56d after cocultivation

Table 6e. Number of *P. padus* leaves producing callus and shoots (in parentheses) on medium supplemented with kanamycin mono-sulphate after cocultivation with *Agrobacterium* C58pMP90 carrying a pBIN19 derivative containing wild-type *NPTII* and *35S::AtAUX1*

Leaves inoculated with	Kanamycin mono-sulphate concentration (mg l^{-1})		
	0	25	Total
+ *35S::AtAUX1*	-	368 (1)	368 (1)
Pseudo-cocultivated	40 (40)	0 (0)	40 (40)
Total	40 (40)	(1)	

Values were derived from two experiments of 20 and 200 replicates for pseudo-cocultivated and + *35S::AtAUX1* inoculated leaves, respectively. Data were recorded 56d after cocultivation
- Indicates that this treatment was not assessed in this experiment

Growth of putative transgenic calluses and shoots

The two calluses produced during *experiment a* (Table *6c*) on explants cocultivated with *Agrobacterium* containing mutant *NPTII* and *35S::AtAUX1* necrosed on transfer to fresh medium of similar composition.

Calluses grew exceedingly slowly, were friable, and varied in colour from dark red to pink, and even white. There were transient increases in the rate at which calluses grew, but repeatedly they necrosed rapidly. In *experiment a*, by 112 d after cocultivation only 27 calluses remained from 95 subcultured every 28 d on medium of similar composition to that on which they had originally formed. Transfer of the remaining cultures to kanamycin-sulphate-free-medium did not improve their growth during eight months of subculturing every 21-28 d, suggesting the effect was not due to KM toxicity. Reducing the subculture period to 7 d (experiment *b*) did not improve growth either as only one of 24 calluses survived three consecutive subcultures. Similar results were observed with experiment *c*. All 24 calluses maintained on medium with 35 mg l^{-1} KM necrosed during four months of subculturing every 21-28 d and 344 calluses maintained on kanamycin-sulphate-free-medium from 56 d post cocultivation exhibited erratic growth and most necrosed over a year of subculturing every 21-28 d.

In experiment *a*, both shoots derived from pseudo-cocultivated explants (Table *6c*) necrosed on transfer to fresh medium of similar composition, whereas the shoot derived from cocultivation with *Agrobacterium* carrying wild-type *NPTII* and *35S::AtAUX1* was propagated (designated line-C2). The shoot produced in experiment *b* (Table *6d*) became contaminated and was lost. Six more putative transgenic shoots developed from calluses derived from experiment *a*, five of which were recovered as shoot lines and designated (with the original concentration of KM selection present in parentheses) line-4, line-10 (both 75 mg l^{-1}), line-C3, line-13 (both 50 mg l^{-1}), and line-19 (25 mg l^{-1}). The sixth failed to proliferate new shoots and necrosed. Eight further putative transgenic shoots were produced from calluses in experiment *c*, seven after transfer to medium without KM, six of which could be propagated, but PCR evidence suggested only one had *35S::AtAUX1*.

In summary, for the three experiments (a-c), from 494 explants which produced calluses after cocultivation with *Agrobacterium* containing wild-type *NPTII* and *35S::AtAUX1*, 17 produced

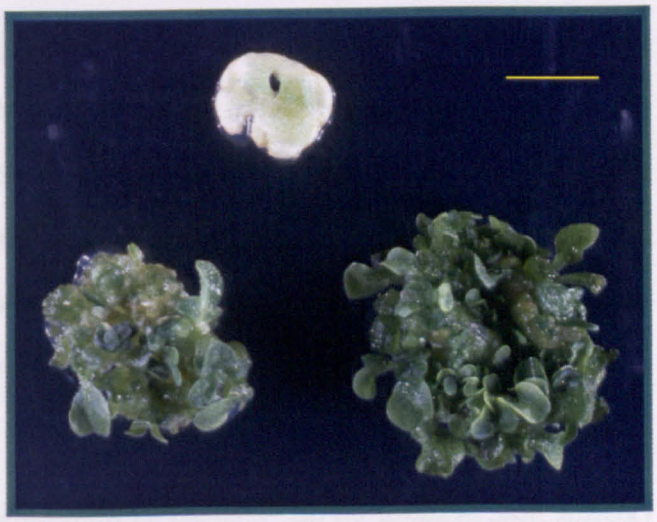
putative transgenic shoots, 12 of which were recovered as shoot lines, and 7 have given a positive PCR result for the presence of *AtAUX1* (Section *6.3.8*).

The inability to produce transformed *P. padus* without *35S::AtAUX1* resulted in the lack of an 'ideal' control for future experiments. To investigate why this occurred and provide suitable control material to support results obtained with *P. padus*, transformation experiments with two easy-to-transform species, tobacco and *Arabidopsis*, were attempted.

6.3.5 *Experiment 6.5: The kanamycin mono-sulphate concentration required to inhibit adventitious shoot regeneration from wild-type Nicotiana tabacum leaf discs*

Forty leaf discs cultured on REM3 without KM produced calluses from which adventitious shoots developed. The number of shoots produced was not recorded since it was impossible to determine accurately whether shoots were adventitious or developed from axillary buds of adventitious shoots. Shoots produced in the absence of KM were green, normal in appearance, and grew rapidly. All leaf discs maintained on medium with 50 mg l^{-1} KM became yellow and necrosed without developing callus or shoots. Thus, a concentration of 50 mg l^{-1} KM was used for the selection of transgenic shoots in subsequent transformation experiments.

Figure 6b. Adventitious shoot regeneration from tobacco leaf discs, after 21 d of culture; (anticlockwise, from top) pseudo- and *Agrobacterium* cocultivated discs on REM3 supplemented with 150 mg l^{-1} kanamycin mono-sulphate, and wild-type discs without selection (scale bar = 10 mm)



6.3.6 *Experiment 6.6 a and b: Transformation of tobacco with 35S::AtAUX1*

Experiment a: All pseudo-cocultivated leaf discs cultured on REM3 without KM produced callus and shoots, but none developed on medium supplemented with 50 mg l^{-1} KM. All 96 leaf discs cocultivated with *Agrobacterium* containing wild-type *NPTII* and *35S::AtAUX1*, maintained on REM3 with or without KM, produced callus and subsequently shoots. However, neither callus nor

shoots were produced on 95 leaf discs cocultivated with *Agrobacterium* carrying wild-type *NPTII* without *35S::AtAUX1*.

From 100 shoots distributed equally amongst CCPs containing TOM1 supplemented with CTX at concentrations from 50 to 150 mg l^{-1} KM a total of 41 independently derived shoot cultures grew. This suggests that a number of these shoots were, as suspected, non-transformed 'escapes'. When the remaining cultures were maintained on TOM1 supplemented with 150 mg l^{-1} KM 15 shoot cultures continued to grow and 12 were retained.

Experiment b: This experiment aimed to provide supporting evidence that a concentration of 150 mg l^{-1} KM would be an appropriate concentration with which to select transgenic shoots.

All pseudo- and *Agrobacterium*-cocultivated leaf discs maintained on REM3 without KM produced callus and subsequently shoots. No pseudo-cocultivated discs produced callus or shoots on REM3 with 150 mg l^{-1} KM, whereas, on this medium 95 % of *Agrobacterium*-cocultivated leaf discs did so (Figure *6b*).

All shoots (from independent regeneration events), derived from pseudo-cocultivated leaf discs maintained on REM3 without KM, necrosed when transferred to TOM1 supplemented with 150 mg l^{-1} KM (n=20). However, of 20 shoots derived from *Agrobacterium*-cocultivated leaf discs (maintained on REM3 with KM) 19 continued to grow on this medium, suggesting they were transgenic and the concentration of KM was appropriate for selection. Fifteen of these putative transgenic shoots were maintained on kanamycin-free TOM1 for analyses (Section *6.3.8*).

6.3.7 Experiment 6.7: Selection on kanamycin mono-sulphate for putative 35S::AtAUX1 transgenic Arabidopsis seedlings

Virtually all T_1 seed (from pseudo- and *Agrobacterium*-cocultivated T_0 plants) germinated on medium with or without 35 mg l^{-1} KM. T_1 seedlings (from T_1 seed) on medium with 35 mg l^{-1} KM had light green 'bleached' appearance, in contrast to the darker green of those maintained on medium without KM. Circa 5 % of T_1 seedlings from plants cocultivated with *Agrobacterium* carrying *35S::AtAUX1* were dark green, whereas, those derived from plants cocultivated with *Agrobacterium* without *35S::AtAUX1* were all bleached. Thus, again transformation with this construct, containing wild-type *NPTII*, failed, raising doubts about the integrity of the construct.

On medium with 50 mg l^{-1} KM, the bleaching of T_2 seedlings (from T_2 seed) gave a segregation ratio consistent with single transgene integration for lines 1, 5, 7, 8, 10, 14 and 20 (Table *6f*), with lines 5 and 10 appearing homozygous.

Table 6f. Resistance to selection on medium with 50 mg l^{-1} kanamycin mono-sulphate of wild-type (wt), and twenty putative *35S::AtAUX1* transgenic *Arabidopsis* T_2 generation seedlings

Seedling line	Resistant	Susceptible	apparent T_2 seed ratio
Wild-type	0	174	0
1	54	18	3 : 1
2	158	32	5 : 1
3	146	6	34 : 1
4	154	22	7 : 1
5	172	0	172 : 0
6	166	40	4 : 1
7	94	30	3 : 1
8	122	38	3 : 1
9	*	*	*
10	98	0	98 : 0
11	90	4	23 : 1
12	*	*	*
13	220	26	8 : 1
14	108	32	3 : 1
15	176	38	5 : 1
16	*	*	*
17	196	4	49 : 1
18	80	6	13 : 1
19	134	12	11 : 1
20	90	28	3 : 1

* indicates where a seed line that was lost due to contamination

Scoring lack of bleaching in T_3 seedlings confirmed that lines 5 and 10 were homozygous, and identified homozygous lines in the remainder. Thus, gave 9 putatively transformed lines with single copies of the *35S::AtAUX1* transgene.

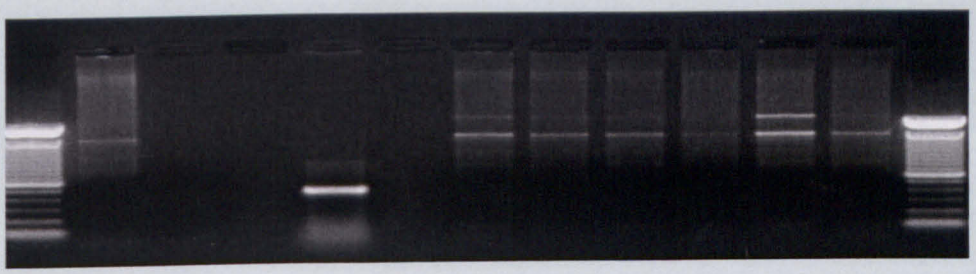
6.3.8 *Polymerase chain reaction (PCR) confirmation of putative* 35S::*AtAUX1 transformants*

No amplification products were seen during PCR analyses from *virDI* primers with DNA extracted from *P. padus* (Figure *6c*) or tobacco (Figure *6d*) shoots, as well as the absence of bacterial colonies from these tissues on YEP medium, suggested that *Agrobacterium* had been eliminated. Analysis of the six independently-derived putative transgenic *P. padus* shoot culture lines with primers for a common sequence within the *35S CaMV* (cauliflower mosaic virus) promoter and sequences within *AtAUX1* suggest that no truncation had occurred during integration of the *35S::AtAUX1* construct into the plant genome. Likewise, similar results were obtained with 15 independently derived putative *35S::AtAUX1* transgenic tobacco culture lines (Figure *6d*). Only one of the putative transgenic shoot lines of *P. padus* recovered from experiment *6.4c* was positive for *35S::AtAUX1* (data not shown).

Figure 6c. PCR analysis of six independent *Prunus padus* shoot lines putatively transformed with the *35S::AtAUX1* construct

Amplification within the virD1 gene

450 bp product



Lanes: 1 2 3 4 5 6 7 8 9 10 11 12 13

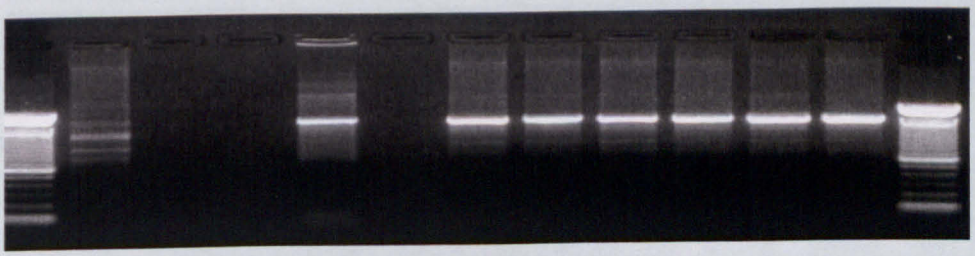
Amplification for $35S_{promoter}$ /AtAUX1 gene

510 bp product

Lanes: 1 2 3 4 5 6 7 8 9 10 11 12 13

Amplification for $35S_{promoter}$ /AtAUX1 gene

1463 bp product



Lanes: 1 2 3 4 5 6 7 8 9 10 11 12 13

100 bp ladder, thicker bands, travelling down the page, represent 2072, 1500 and 600 bp

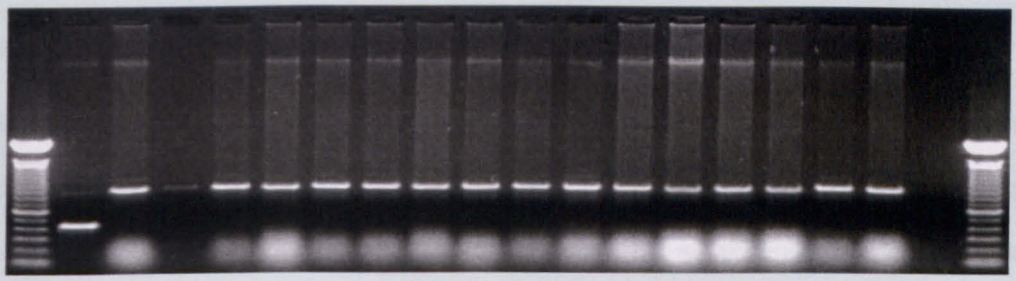
Key for content of gel lanes:

1. Ladder	5. *Agrobacterium* + *35S::AtAUX1*	9. 4	13. Ladder
2. Wild-type *P. padus*	6. Empty	10. 10	
3. Reagents only	7. C2	11. 13	
4. Empty	8. C3	12. 19	

Figure 6d. PCR analysis of fifteen independent tobacco shoot lines putatively transformed with the *35S::AtAUX1* construct

Amplification within the virD1 gene

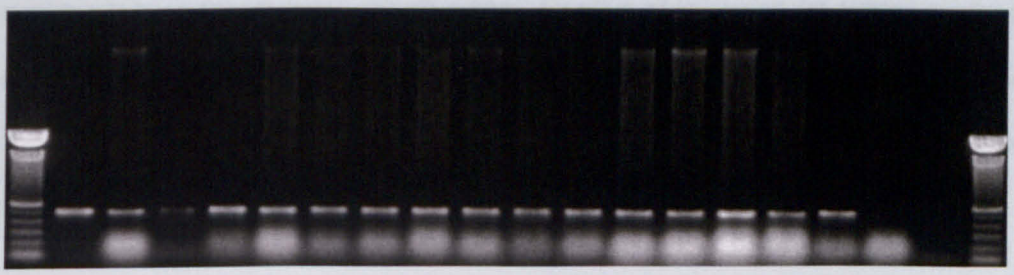
450 bp product



Lanes: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Amplification for $35S_{promoter}$ /AtAUX1 gene

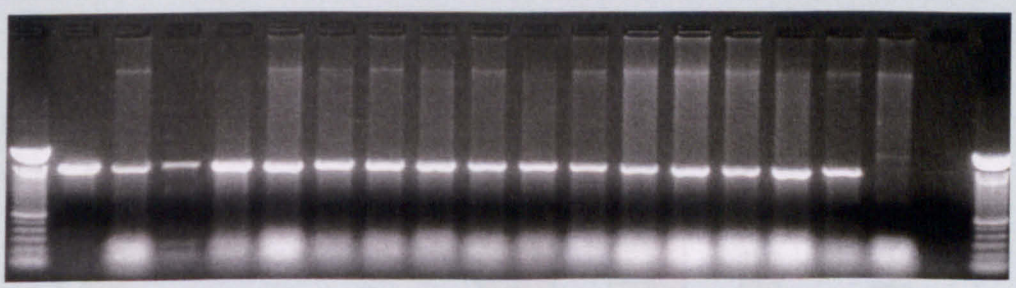
510 bp product



Lanes: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Amplification for $35S_{promoter}$ /AtAUX1 gene

1463 bp product



Lanes: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

100 bp ladder, thicker bands, travelling down the page, represent 2072, 1500 and 600 b p

Key for content of gel lanes:

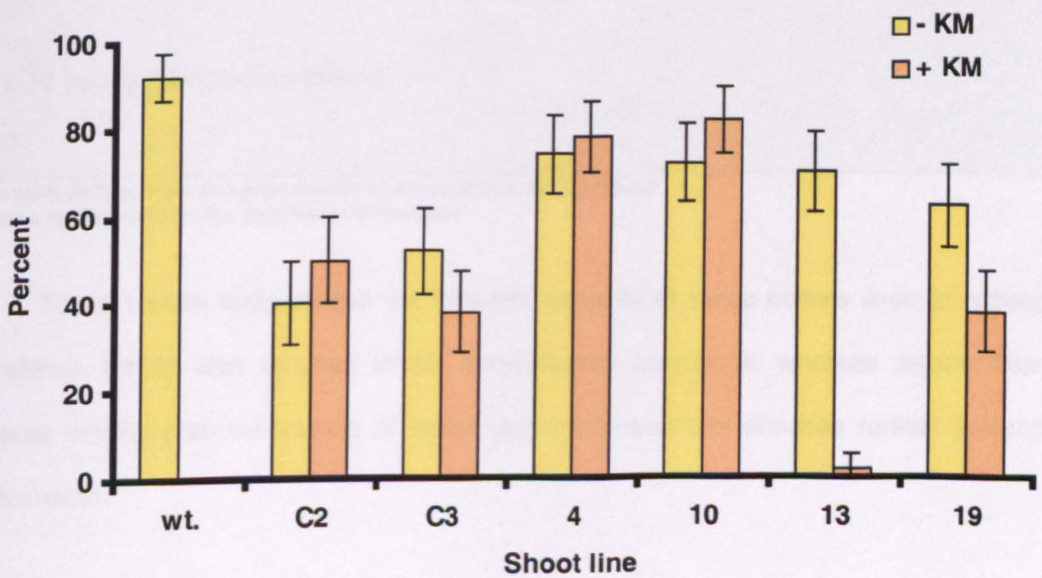
1. Ladder	6. D	11. I	16. N
2. *Agrobacterium* + *35S::AtAUX1*	7. E	12. J	17. O
3. A	8. F	13. K	18. Wild-type tobacco
4. B	9. G	14. L	19. Reagents only
5. C	10. H	15. M	20. Ladder

6.3.9 *Experiment 6.8: The adventitious shoot regeneration capacity of 35S::AtAUX1 transgenic P. padus leaves*

This experiment tested the hypothesis that *35S::AtAUX1* transgenic *P. padus* had reduced capacity to regenerate shoots as an explanation for the low rates of regeneration from putative transgenic cultures.

On REM2, 92 % ± 5.4 (± se.) of wild-type leaves produced shoots (Figure *6e*), but none developed on medium supplemented with 25 mg l^{-1} KM. The proportions of leaves producing shoots from *35S::AtAUX1* transgenic lines-4, 10 and 13 were not significantly (P=0.05) different from that of wild-type leaves on medium without KM, but were lower for lines C2 (P<0.001), C3 (P<0.01), and 19 (P<0.05). Relative to wild-type leaves on medium without kanamycin mono-sulphate, transgenic lines C2 (P<0.01), C3 (P<0.001), 13 (P<0.001) and 19 (P<0.001) produced fewer shoots on medium with KM, whereas lines 4 and 10 were not significantly different. For individual transgenic lines, with the exception of line 13 (P<0.01), the inclusion of KM did not reduce the proportion of leaves producing shoots.

Figure 6e. The proportions of wild-type (wt) and 35S::*AtAUX1* transgenic *Prunus padus* leaves that produced adventitious shoots on REM2 with or without 25 mg l^{-1} kanamycin mono-sulphate (KM)



Values were derived from two experiments of 25 replicates per treatment (± SE)
Data were recorded 56 d after treatment commenced

The mean mass of wild-type culture (Table *6g*) on REM2 supplemented with KM was significantly lower than that of wild-type cultures on medium without KM (P <0.001). Relative to wild-type cultures on medium without KM, the mass of all *35S::AtAUX1* transgenic lines was lower (P<0.01) on medium with KM, except for line 10, which was not significantly different (P=0.05). For individual transgenic lines, the inclusion of KM did not influence culture mass for lines C3, 4 and 10 (all P <0.05), but the mass of culture lines C2 (P <0.01), 13 (P <0.001) and 19 (P <0.001) was lower.

Table 6g. Fresh mass (mg) of wild-type (wt.) and *35S::AtAUX1* transgenic *Prunus padus* cultures maintained on REM2 with and without 25 mg l^{-1} kanamycin mono-sulphate

Transgenic shoot line	Kanamycin mono-sulphate concentration (mg l^{-1})	
	0	25
wt.	368 (5.908)	24 (3.177)
C2	217 (5.382)	118 (4.771)
C3	103 (4.639)	81 (4.396)
4	234 (5.454)	199 (5.295)
10	338 (5.824)	410 (6.017)
13	247 (5.509)	48 (3.875)
19	144 (4.968)	96 (4.566)
SEM. 0.10 (for log values in parentheses)		
d.f. 113		

Values were derived from two experiments of 25 replicates per treatment
Data were recorded 56 d after treatment commenced

These results suggest that the inherent capacity of some culture lines to regenerate adventitious shoots was reduced under non-selective conditions, whereas regeneration on otherwise inhibitory concentrations of kanamycin mono-sulphate provides further evidence of transformation.

6.4 Discussion

Agrobacterium-mediated DNA delivery is often less technically exacting than micro-projectile- or protoplast-based systems and can produce intact, low copy number, non-rearranged transgene integration (Gelvin, 1998). Consequently, I thought this approach would offer the most expeditious route. However, the choice of transformation vector and aminoglycoside antibiotic-based selection for this study were dependent on available technology and a compromise between the time required to develop and test new vectors against the need for rapid progress. Possible improvements are discussed elsewhere (Section *6.4.5*).

6.4.1 Attempted transformation of P. avium with 35S::AtAUX1

The concentration of kanamycin mono-sulphate required to inhibit shoot regeneration from wild-type *P. avium* leaves proved difficult to determine because of experiment-to-experiment variation. Nevertheless, an attempt was made to transform accession 1905, which, with 1906, had produced greater numbers of adventitious shoots per leaf than eight other arbitrarily selected *P. avium* genotypes (Chapter *5*). However, this was unsuccessful. The inability to produce putative transgenic callus or shoots (that could be maintained) was disappointing, but perhaps not surprising due to there being such a short period where *P. avium* leaves could be induced to form shoots.

Sixty percent of pseudo-cocultivated and a similar proportion of *Agrobacterium*-cocultivated leaves regenerated shoots on non-selective medium. Thus, leaves were competent for regeneration before selection and unaffected by cocultivation conditions

The inability to produce transgenic callus and regenerate adventitious shoots may not result from failure of T-DNA integration. Previous studies with *A. tumefaciens* strain C58pMP90 carrying pBIN19 with either *β-glucuronidase* (*GUS*) containing an intron (Vancanneyt *et al.*, 1990) or an 11.4 kb *Xba*I fragment of the T-DNA encompassing the oncogenes of wild-type *A. tumefaciens* plasmid pTi82.139 (Michel *et al.*, 1990) suggest this species can be transformed by *Agrobacterium*, at least with marker genes under non-selective conditions (Grant *et al.*, 1998). With the latter construct, the virulence of *Agrobacterium* strains can be ascertained independently from the influence of the T-DNA, as each strain is engineered with a common T-DNA carrying plasmid (pT139). The authors used this procedure to establish the relative

transformation efficiency on *P. avium* by *A. tumefaciens* strain 82.139, and disarmed LBA4404, ALG1, GV2260 and C58pMP90 strains which were rearmed with pT139. They concluded that a C58pMP90 background gave superior transformation rates and that the virulence genes contributed to the relative differences in transformation efficiency. Thus, *Agrobacterium* strain C58pMP90-based constructs were shown to be proficient at transforming *Prunus*. However, Lowe *et al.* (1992) hypothesised that tumour formation by wild-type strains may be unreliable as an indicator of the relative ability of related disarmed strains to mediate transformation of *Dendranthema grandiflora*. Part of their study involved electroporation of a plasmid containing the T-DNA of wild-type *Agrobacterium* strain A6, which was virulent towards *D. grandiflora*, into a disarmed strain of LBA4404 (derived from wild-type strain ACH5), which was non-virulent towards *D. grandiflora*), but contained active virulence genes. The resulting *Agrobacterium* could transform *D. grandiflora* with similar efficiency to strain A6, suggesting the T-DNA/oncogenes moiety can influence transformation efficiency. It is probable that the endogenous growth regulator profile of transgenic cultures expressing the oncogenes of *Agrobacterium* strain 82.139 would differ from that achieved where growth regulators were applied exogenously. If expression of the oncogenes produced a cytokinin and auxin combination particularly effective at eliciting the growth and development of transgenic cells/tissues this may explain why transgenesis *of P. avium* could not be achieved in *P. avium* by constructs lacking these genes. However, transient *GUS* expression had been achieved previously without the aid of a construct containing oncogenes, although under non-selective conditions (Grant *et al.*, 1998).

Maximova *et al.* (1997), investigating *Agrobacterium*-mediated transformation of four apple cultivars with a SGFP (synthetic green fluorescent protein) marker, observed a 10,000-fold decrease in shoot regeneration under selective conditions. From 100-500 transformed cells per explant 48 h after commencing cocultivation, only 1-5 developed into fluorescing calli after 14 d under selective conditions, indicating inefficient T-DNA integration into the host genome or subsequent gene silencing. Under selective conditions, failure of *P. avium* explants to develop transgenic calluses and shoots may have a similar basis, especially if a low number of cells were transformed initially. Significantly different patterns of shoot regeneration were also observed under non-selective and selective conditions in apple (Maximova *et al.*, 1997). Adventitious shoots formed without an intermediate callus phase under non-selection conditions, whereas

regeneration occurred via a callus phase, at a much slower rate, under selective conditions. Conceivably, shoot primodia may develop from multiple cells under non-selective conditions, whereas, antibiotic selection may force shoot primodia to develop from a single founder cell, or a much reduced population of cells. It is possible that the tissue culture conditions may not have been conducive to such a pattern of development for *P. avium*. However, this does not explain the lack of callus formation unless failure of shoot meristem primordia elaboration inhibited the overall development of calluses.

Provided individual cells were both transformation/regeneration competent, deferring the beginning of selection until cells were committed to an adventitious shoot primordia developmental pathway may have improved the recovery of transgenic shoots. However, this may have increased risk of producing chimaeric plants. Alternatively, regenerating shoots without (or with deferred) selection, followed by regeneration, under selective conditions, from leaves derived from these putative chimaeric plants may have been a better option. The rationale is that a larger pool of transformed cells, potentially more resistant to selection, could perhaps be preferentially recruited into forming an adventitious shoot primordia.

6.4.2 Production of 35S::AtAUX1 transgenic Prunus padus

The capability to induce relatively large numbers of adventitious shoots under non-selective conditions and, of primary importance, the potential to regenerate shoots from callus suggested that this species may be easier to transform than *P. avium*. Furthermore, since the primary objective was to develop a *Prunus*-based woody model for the investigation of adventitious rooting, I took the decision to focus all further effort on the transformation of *P. padus*, rather than attempt to resolve the problems associated with *P. avium* transformation.

Although not compared in the same experiment, results suggest the concentration of kanamycin mono-sulphate required to prevent regeneration from wild-type leaves was lower for this species than with *P. avium*. Thus, variation in tolerance to kanamycin mono-sulphate toxicity may occur within the genus.

Across all treatments, 97 % (348 of 360) of explants maintained on kanamycin-free-medium regenerated shoots, suggesting conditions were conducive for regeneration before selection was applied, and unaffected by cocultivation.

The hypothesis that a mutation within the *NPTII* gene would reduce transformation efficiency under kanamycin mono-sulphate selection was investigated in *P. padus*, using constructs, with and without this defect, either in the presence or absence *35S::AtAUX1*. Initial results appeared to support this hypothesis, since in the presence of the *35S::AtAUX1*, callus formation occurred only where the construct contained wild-type *NPTII* (i.e., pRD400*AUX1*). Likewise, cocultivation with Agrobacteria containing the mutant *NPTII* (i.e., pBIN19 and pROK2*AUX1*) did not produce callus. However, the complete absence of callus formation where the construct contained wild-type *NPTII* without *35S::AtAUX1* (i.e., pRD400) is difficult to explain, being suggestive of synergism between these genes.

From a total of 600 leaves cocultivated with *Agrobacterium* containing *35S::AtAUX1* and wild-type *NPTII*, which were maintained under selective conditions, 534 produced calluses (89 %). However, it was extremely difficult to maintain these habitually friable calluses and prevent necrosis. Moreover, the capacity of these cultures to produce adventitious shoots was severely reduced compared to that of wild-type cultures. It is improbable that these attributes resulted from positional effects of transgene insertion, because of the large number of independently transformed calluses affected. Furthermore, it is enigmatic that expression of *35S::AtAUX1* appeared to promote transformation efficiency initially and subsequently inhibited the growth and development of putative transgenic calluses and shoot regeneration capacity.

It is possible that cells competent for transformation were not the same cells competent to regenerate shoots and *vice versa*. For example, under kanamycin mono-sulphate selection, Geier and Sangwan (1996) observed that adventitious shoots originated from the epidermis of *Kohleria* internode explants, but cells competent for *Agrobacterium*-mediated transformation predominated in the vascular tissue. Lowe *et al.* (1992) reported similar disparities between cellular competence for these processes in *D. grandiflora*. However, transformable cells being incompetent for regeneration, or a shift in mode of regeneration under selective conditions (discussed previously for *P. avium*), inadequately explain the data; although, the former could possibly explain the low frequency of shoot regeneration from putative transgenic calluses.

The effects on caulogenesis and organogenesis were, at least partially, independent of 'selection pressure', occurring either in the presence or absence of kanamycin mono-sulphate. In experiment *6.2a*, the proportion of cultures with calluses, or the mean number of calluses

produced per explant were unaffected by kanamycin mono-sulphate concentration. However, calluses subcultured to kanamycin-free-medium still maintained erratic patterns of growth and failed to regenerate shoots. Data from experiment *6.8* suggests, that for individual transgenic lines, with the exception of line 13, selection on 25 mg l^{-1} kanamycin mono-sulphate did not influence the proportions of excised leaves producing adventitious shoots, although the mass of culture lines C2, 13 and 19 were significantly reduced. However, relative to wild-type cultures, data from this experiment also indicated that leaves from three (i.e., C2, C3 and 19) of the six transgenic shoot lines had significantly reduced capacity to form adventitious shoots on kanamycin-free-medium. Thus, the inherent competence of these cultures to grow and regenerate adventitious shoots may be compromised by the activity of the putative auxin influx protein, AtAUX1.

Polar auxin transport may be necessary for the normal development of shoot primordia and meristems. Thus, activity of the AtAUX1 protein may disrupt the mechanism(s) eliciting adventitious shoot regeneration. Nevertheless, meristems were present on *35S::AtAUX1* transgenic shoot cultures from which leaves were excised in experiment *6.8*. Possibly, the acquisition of cellular competence (i.e., responsiveness to an inducing stimuli) and/or the induction of cellular determination (i.e., commitment to a specific developmental fate), which has to occur for *de novo* meristem formation may be more sensitive to disruption than in pre-existing meristems.

Conceivably, disruption of auxin transport may have occurred if cells, which normally took an insignificant role in auxin transport, were recruited into taking up auxin when *AtAUX1* was constitutively expressed under a *35S* promoter. Cells may have acted as sinks for auxin, reducing the auxin gradient and/or dissipating polarity. Alternatively, callus necrosis and reduced organogenic capacity may have resulted from intracellular auxin concentration becoming phytotoxic in *35S::AtAUX1* expressing cells. Determination of total auxin content and the transport velocity of radio-labelled auxin in stem sections from wild-type and transgenic shoots may resolve this issue. However, subtle redistribution of auxin may be undetectable.

To improve transformation efficiency, it may be necessary to deactivate *AtAUX1* until transgenic shoots are recovered by limiting expression temporally by placing the gene under the control of an inducible promoter [e.g., Dexamthasone; Aoyama and Chua (1997)]. Alternatively,

expression could be focused in particular target cells/tissues [e.g., *rolB and rolC* promoters which have been found to be predominantly expressed in pericycle cells which serve as root initials in transgenic hybrid aspen; Nilsson *et al.* (1997)].

6.4.3 Production of 35S::AtAUX1 transgenic tobacco and Arabidopsis

The anomalies encountered during the transformation of *Prunus* were investigated further by transgenesis of *Nicotiana* and *A. thaliana*, two easy-to-regenerate species. Supplementing medium with 50 mg l^{-1} kanamycin mono-sulphate completely inhibited adventitious shoot regeneration from wild-type tobacco leaf discs, but a high regeneration frequency implied this was ineffective at preventing untransformed shoots from regenerating during subsequent transformation experiments. This result, and the difficulty encountered with *P. avium*, suggests that it may have been more appropriate to determine the concentration of kanamycin mono-sulphate required for selection under transformation conditions. Nevertheless, a number of putative *35S::AtAUX1* transgenic tobacco lines were isolated during a second round of selection on medium supplemented with 150 mg l^{-1} kanamycin mono-sulphate (experiment *6.6b*). However, there is always the possibility that inadequate selection pressure would result in the formation of chimaeric shoots, as shown by Schmulling and Schell (1993) where tobacco transformed with a *rolC* gene as a visual marker formed periclinal chimeras (i.e., genetically different cell populations in different cell layers).

Transgenic cultures were not produced after the cocultivation of *Nicotiana* or *A. thaliana* tissues with Agrobacteria containing wild-type *NPTII* (i.e., pRD400), but callus and shoots developed where the construct contained *35S::AtAUX1* and wild-type *NPTII* (i.e., pRD400*AUX1*). The reproducibility of the results obtained with *P. avium* and *P. padus* in these easy-to-transform species suggests there may be a problem with this stock of plasmid pRD400. Furthermore, failure to induce transgenesis from *Nicotiana* leaf discs cocultivated with *Agrobacterium* C58pMP90 carrying new constructs based on this plasmid (data not shown), which contained the cherry homologue of *AtAUX1* (*CHAX1*) or *GUS*-intron genes driven by either *rolB and rolC* promoters endorses the suspicion of a defective plasmid. However, all constructs based on this plasmid were checked and found not to contain the known *NPTII* gene mutation, although it is possible there may be another unknown mutation within the gene. Plasmid pRD410 was used instead of pRD400 in the construction of pRD400*AUX1* because the *AtAUX1* sequence

contained a restriction site which would have been required for insertion into pRD400. Theoretically, plasmids pRD400 and pRD400*AUX1* were identical except that the latter contained *AtAUX1* with associated promoter and terminator sequences. Thus, there are several hypotheses which could explain these results. Conceivably, pRD400 may be dysfunctional, or simply not actually a pBIN19 derivative. Alternatively, pRD410, after the removal of the *GUS*-intron gene may not in fact be equivalent to pBIN19 (plus the retained *CaMV35S* promoter) and in some unknown way this difference may have influenced transformation efficiency. Possibly, sequencing and comparing plasmids pBIN19, pRD400 and pRD400*AUX1* with the known data base sequence for pBIN19 may have resolved this problem.

Unfortunately time constraints precluded further molecular characterisation and investigation of the rooting phenotype of transgenic tobacco or *Arabidopsis*. However, neither species provided transformed shoots without *35S::AtAUX1* which would have provided the control material necessary to support conclusions drawn from *P. padus*.

6.4.4 Problems associated with confirming transformation

There are various strands of evidence [i.e., caulogenesis and organogenesis in the presence of kanamycin mono-sulphate concentrations normally inhibitory to growth (this Chapter), reduced rooting capacity of putative transgenic shoots and an inability to mimic this rooting phenotype using shoots derived from cultures established from adventitiously regenerated wild-type shoots (Chapter 7)] suggesting that *35S::AtAUX1* transgenic *P. padus* cultures were produced. PCR analyses support this conclusion. However, the molecular evidence remains incomplete, requiring further confirmation by Southern blotting and either northern or RT-PCR analyses to assess levels of expression.

Extraction of DNA from *Prunus* tissues proved extremely difficult. Woody plants can contain relatively high concentrations of products (e.g., proteins, polyphenols and polysaccharides) able to degrade nucleic acids, impede purification and inhibit subsequent manipulations (e.g., PCR and endonuclease digestion). Purity of DNA can be determined by spectrophotometry with the ratio of absorbance at A_{260}/A_{230} and A_{260}/A_{280} nm as a measures of contamination by polyphenols/carbohydrates and proteins, respectively (Manning, 1991). Appropriate ratios are 1.7-1.9 for the former and >2 for the latter. Typically values obtained with *P. padus* were <1-1.5 and circa 1.5 for absorbance at A_{260}/A_{230} and A_{260}/A_{280} nm, respectively.

Several methods were evaluated for the rapid extraction of DNA for PCR analyses, including those based on sodium dodecyl sulphate (Edwards *et al.*, 1991) or alkali lysis (Klimyuk *et al.*, 1993) which gave low yields and poor reproducibility. However, cetyltrimethylammonium bromide (CTAB) based methods have extracted high quality DNA from several woody species [e.g., Doyle and Doyle (1990), Stewart and Via (1993), Lodhi *et al.* (1994)]. Polyvinylpyrrolidone (PVP-40) via entrapment by hydrogen bonding and β-mercaptoethanol by inhibiting oxidation prevent the formation of insoluble phenolic complexes that can co-precipitate with DNA. Modification of the Doyle and Doyle (1990) protocol by increasing CTAB to 4 % (w/v), β-mercaptoethanol to 1 % (v/v) and the addition of 1 % (w/v) PVP-40 permitted extraction of DNA suitable for PCR analyses.

To facilitate chemical extraction and improve DNA yields, mechanical reduction of plant tissue is usually required. Vortexing with glass bearings, a similar approach to that taken by Colosi and Schaal (1993) was relatively ineffective at disrupting cherry tissues. However, freezing tissues with liquid nitrogen and co-grinding with aluminium oxide was effective. Employing 'single use' disposable equipment throughout the extracting protocol effectively eliminated cross contamination.

Neither the original or modified Doyle and Doyle (1990) protocols provided DNA in sufficient quantity or quality for Southern analyses and several additional techniques were investigated. For a number of species, higher sodium chloride concentrations than used by Doyle and Doyle (1990) have improved the solubility of polysaccharides in ethanol and prevent their co-precipitation with DNA during CTAB-based extraction [e.g., *Vitus* spp., Lodhi *et al.* (1994); *Sedum telephium*, Barnwell *et al.* (1998)]. Nevertheless, these modifications were ineffective with *P. padus* as were non-CTAB-based protocols [e.g., 2-butoxyethanol, Manning (1991); SDS/Proteinase K, Gerlach and Stosser (1997); PVP-40/SDS/boric acid], used either directly or as methods to clean up CTAB-derived nucleic acids. Electrophoresis of contaminated DNA through low melting point agarose and subsequent recovery with 'GELase' agarose digesting enzyme (Cambio, UK) reduced the contamination, but restriction digests were still inhibited. Purification by equilibrium centrifugation in CsCl was also attempted but DNA could not be recovered. Plant DNA extraction kits, 'Nucleon' (Phytopure, USA) and 'DNeasy 96' (Qiagen, UK)

were also ineffective. Extraction of RNA proved just as problematic as for DNA precluding their use as substrates in RT-PCR analyses.

It is difficult to see how the problem of extracting good quality nucleic acids can be resolved. Possibly, carbohydrates are being loaded into *in vitro* grown material, suggesting extraction from *ex vitro* material may be better. However, it has been extremely difficult to transfer transgenic plants to soil, although this was achieved with lines 4 and C3 the plants failed to survive subsequent over-wintering. Reduction of total carbohydrate concentration or use of an alternative carbon source during the tissue culture of *P. padus* may be helpful but may have ramifications on the quality and growth of cultures, which may be detrimental to nucleic acid extraction. Dark-grown callus cultures may have reduced concentrations of polyphenols and may provide cleaner extracts of nucleic acids. Alternatively, partial lysis of the cell to leave intact nuclei which can be partitioned from the other cell debris and contaminates, before undergoing further purification, may be worth investigating.

6.4.5 Improving the transformation strategy

Public concern about the perceived/actual risks associated with genetically modified organisms [Ruibal-Mendieta and Lints (1998), Flavell (2000)] and scientific imperative demand circumspection and the use of 'clean technology' to assure continuance of research. Therefore, plasmid pBIN19 and its derivatives (e.g., plasmids pRD400 or pBINplus) are unsuitable vectors for further work.

To date, scientific data suggest there are no health or safety reasons to restrict the use of neomycin transferase II as a selectable marker [Nap *et al.* (1992), Fuchs *et al.* (1993)], but this may be untrue for other marker genes necessitating expensive risk assessments and delaying the release of transgenic products. Furthermore, retention *in planta* of these finite genes precludes their subsequent use in recurrent transformations of that plant. Consequently, removal of superfluous marker/selection genes from regenerated transgenic plants is highly desirable in future transformation strategy. It is possible to eliminate these genes by sexual crossing (Yoder and Goldsbrough, 1994). However, this approach is unsuitable if species have a long generation time, require vegetative propagation, are sterile or if several transgenes were inserted independently, segregation may result in their loss in subsequent generations. Therefore, these methods are not applicable to the transformation of trees. Strategies not reliant on sexual

crossing have been devised [Ebinuma *et al.* (1997), Sugita *et al.* (1999, 2000), Gleave *et al.* (1999)]. However, all have disadvantages at present and will require further work to improve their acceptability.

The system devised by Gleave *et al.* (1999) is based on integration of *lox*P flanked selection/marker and *codA* genes (encoding cytosine deaminase which converts 5-fluorocytosine to 5-flourouracil to 5-fluroouracil, a precursor of 5-fluro-dUMP which irreversibly inhibits thymidylate synthase depriving the cell of dTTP) during a primary round of transformation followed by transient expression of a non-integrated *cre*-recombinase gene to mediate excision. Selection against transgenic tissues/shoots retaining marker genes is on medium containing 5-fluorocytosine. Drawbacks, reported in tobacco, are unwanted low frequency stable integration of the *cre*-recombinase gene and the requirement for consecutive transformations. Furthermore, transformation efficiency even in this relatively easy-to-transform species was low (0.25 %).

The 'MAT-vector' (multi-auto-transformation) system [Ebinuma *et al.* (1997), Sugita *et al.* (1999, 2000)] is based on an *ipt* gene encoding isopentenyl-transferase which catalyses the condensation of isopentenyl-pyrophosphate and adenosine-5-monophosphate to isopentenyl-adenosine-5-monophosphate, the first intermediate in cytokinin biosynthesis. Overexpression of *ipt in planta* elevates endogenous cytokinin concentration, producing a 'shooty' phenotype with aberrant morphology (e.g., excessive caulogenesis, reduced internode elongation, inhibited adventitious rooting capacity and the ability to proliferate on growth-regulator-free culture medium). These traits previously prohibited the use of *ipt* as a selectable maker since the aberrant phenotype remained with the transgenic plant, a problem rectified by this system.

In the current MAT-vector system (Sugita *et al.*, 2000), transgenic shoots are identified by aberrant morphology and if required *GFP* or *GUS* markers. Removal of the *ipt* gene, with associated recombinase and other selection/marker genes, which are delimited by directly orientated recombination sites, is mediated by a site-specific recombination system *R*/RS from *Zygosaccharomyces rouxii*. Marker-free transgenic shoots result when reintegration fails to occur and the aberrant morphology reverts to wild-type. The transgene(s) conferring the desired trait(s) are independent within the T-DNA and unaffected by excision of the *ipt* containing array. The *R* recombinase is driven by the herbicide antidote 'Safener' inducible glutathione-S-transferase (GST-II-27) promoter from *Zea mays*, which reduces premature excision of *ipt* and promotes the

recovery of transgenic shoots with single T-DNA inserts at high frequency. However, site-specific recombination systems leave a 'footprint' within the host genome after excision as one recombination site remains. Theoretically, chromosomal rearrangements could occur between this site and a second excised site if it were located nearby; the limitations this places on subsequent transformations requires further investigation.

Presently, antibiotic and herbicide based selection for transgenic tissues predominate being reliant on negative selection, whereby non-transgenic cells necrose under the phytotoxic regime of the selective agent to leave transformed cells. However, positive selection methods [e.g., Sugita *et al.* (2000), Aoyama and Chua (1997), Haldrup *et al.* (1998)], which actively promote the growth of transgenic cells may increase transformation efficiency. They are also free of many constraints inherent to antibiotic and herbicide based systems. Haldrup *et al.* (1998) used the xylose isomerase gene (*xylA*), from *Thermoanaerobacterium thermosulpherogens* to enable transgenic plants to utilise xylose as a carbon source (in conjunction with low sucrose) as a method of selection. Higher transformation efficiencies were achieved using this method than with kanamycin mono-sulphate based selection for potato and tomato, but efficiency with tobacco was not significantly different. However, this system has not been evaluated in difficult to transform species, such as cherry.

Ideally, vectors should contain only the DNA to be transferred to the host plant and the sequences required for this to be achieved. Pervasively, it had been assumed that only sequences bounded by the T-DNA borders were integrated into the hosts genome. However, a number of studies [e.g., Ramanathan and Veluthambi (1995), Kononor *et al.* (1997)] suggest vector backbone sequences, flanking both the left and right T-DNA borders, can be integrated during *Agrobacterium*-mediated transformation. This presents an obstacle to the use of many of the vectors presently available and highlights a need for new 'safer' vectors. The use of reduced DNA 'cleaner' vectors such as pGreen (Hellens *et al*, 2000) in which erroneous or redundant sequences no longer flank the T-DNA region, or encompassing the T-DNA with genes [e.g., *barnase*; Hanson *et al.*, (1999)] which encode phytotoxic products are possible strategies for reducing the risks associated with, or the occurrence of, illegitimate integration of deleterious backbone sequences.

The insoluble fibrillar network constituting the nuclear matrix is hypothesised to function in nuclear organisation with the higher-order structure of chromatin mediated by DNA sequences, matrix attachment regions (MARs), that bind this matrix (Homes-Davis and Comai, 1998). Although the specific mechanism is unclear, MARs may act as DNA loop anchors or have a regulatory role in facilitating the transition between closed and open chromatin domains to stabilise gene expression. The rate of transcription from transgenes has been postulated to depend on the chromatin domain into which T-DNA integration occurs (Gelvin, 1998). Delimiting transgenes with MARs has reduced expression variability to levels attributable to environmental and developmental influences (Mlynarova *et al.*, 1996). Han *et al.* (1997) used MARs to enhance transgene expression, and transformation efficiency, in a transformation recalcitrant poplar clone (*Populus trichocarpa* x *P. deltoides*). Possibly, incorporation of MARs may counter any reduction in transformation efficiency caused by flanking the T-DNA with 'lethal genes' as a method to stop backbone insertion.

In summary, an improved transformation strategy for cherry should use a 'clean' vector, possibly the best of these currently available is pGreen (Hellens *et al*, 2000), with *ipt*-based dominant selection of the MAT system (Sugita *et al.*, 2000) for the delivery of marker-free transgenic plants. Possibly, positive selection based on elevated endogenous cytokinin production would benefit species, such as *P. avium* and *P. padus*, which seem recalcitrant to transformation with antibiotic-based negative selection. Expression of *AtAUX1* should be regulated by either tissue-focused promoters (i.e., *rolB* or *rolC*) or the dexamthasone inducible promoter (Aoyama and Chua, 1997). Kunkel *et al.* (1999) demonstrated that this latter promoter gave tight regulation of the *ipt* gene. Furthermore, flanking the *AtAUX1* construct with MARs (Han *et al.*,1997), and the T-DNA by *35S*-driven *barnase* genes (Hanson *et al.*, 1999), should be investigated.

7. Adventitious Rooting of *35S::AtAUX1* Transgenic *Prunus padus* Shoots

7.1 Introduction

The capacity to induce adventitious rooting is of economic importance (Section *1.1*), and many techniques have been employed to enhance the success of propagation by cuttings (Chapters *1*, *3* and *4*). The molecular genetic investigation of the basis of rooting competence (Section *1.5.1*), or more precisely lack of competence, the horticulturally important factor, has, to date, focused on monogenic mutants perturbed in their capacity to perceive and/or modulate response(s) to exogenous and/or endogenous auxin (Section *1.5.2*). However, inadequate knowledge of the fundamental mechanism(s) involved, remains a significant obstacle to the adventitious rooting of many woody species, and invites novel approaches to investigate and/or improve the process: transgenesis (Chapter *6*) provides one such approach.

The adventitious rooting capacities of several woody species have been genetically modified. Infection by *Agrobacterium rhizogenes* can promote adventitious root formation at the site of inoculation, as a result of the stable integration of T-DNA (Section *6.1.3*) into the host's genome and the expression of genes encoded within this region (Chilton *et al.*, 1982). Suspensions of *A. rhizogenes* have been applied to cuttings of rooting recalcitrant genotypes of walnut and have triggered successful rhizogenesis [Caboni *et al.* (1996); Giuseppina *et al.* (2000)]. Within *A. rhizogenes* T-DNA, 18 open reading frames have been identified (Slightom *et al.*, 1986), and root loci (*rol*) A, B, C, and D are reported to be involved in promoting rhizogenesis (Vilaine *et al.*, 1987), with *rolB* having the greatest promotory effect (Capone *et al.*, 1989). Results of Delbarre *et al.* (1994), with *rolB* transgenic tobacco protoplasts, suggest that the *rol*B protein enhances sensitivity to auxin by influencing the auxin perception pathway. Tyrosine phosphatase production, localised to the plasma membrane, in transgenic plants suggests a role for the *rol*B protein in the transduction of the auxin root-inducing signal (Filippini *et al.*, 1996). Consequently, transgenic aspen - *Populus tremula* (Tzfira *et al.*, 1998), expressing *rol*B and *rol*C, and the apple rootstock M26 - *Malus* x *domestiica* Borksh. (Welander *et al.*, 1998) expressing *rol*B, all controlled by native promoters, exhibited enhanced sensitivity to auxin and improved rooting.

Maturation is a key factor influencing adventitious rooting capacity in woody species (Chapter *1* and *3*). The endogenous concentration/composition of flavonoids has been found to vary between development phases in species such as Ivy (*Hedera helix*; Murray *et al.*, 1994) and walnut (*Juglans nigra* x *juglans regia*; Claudot *et al.*, 1992). Flavonol glycosides (e.g., myricitrin and quercitrin) were postulated to cause inhibition of rooting in walnut (*Juglans nigra* x *juglans regia*), possibly, via disruption of H^+ ATPase activity and/or the transport mechanisms of auxin and/or sucrose (Claudot *et al.*, 1992). Further evidence implicating a role for endogenous flavonoids in the regulation of auxin efflux (Section *1.4.7*) *in planta* were obtained with the flavonoid-deficient mutant of *A. thalaina*, *tt4* (Murphy *et al.*, 2000), including, a stated increase in lateral root formation resulting from flavonoid reduction (data not published). Expression of antisense chalcone synthase, under a double *35S* promoter, reduced the tissue content of this key enzyme in flavonoid biosynthesis, and that of the flavonol glycosides, improving rooting of walnut shoots *in vitro* (El Euch *et al.*, 1998).

Although, not the only factor (Chapter *1*), the efficacy of auxin as a promoter of adventitious rooting is compelling (Haissig and Davis, 1994), and that an association may exist between endogenous auxin concentration and rooting capacity is plausible (Blakesley, 1991). Further, the evidence given above demonstrates a prevalent theme in which the manipulation of endogenous auxin has modified rooting capacity. Evidence (Chapter *1.5.1*) exists suggesting that rooting capacity may depend on cellular competence to perceive and/or respond to the root-inducing stimuli, postulated to be auxin.

Auxin redistribution *in planta* from sites of biosynthesis and exogenous application is hypothesised to be obligate upon a component of active transport; inhibition of this predominantly basipetal polar auxin transport flux has profound effects on plant development including rooting (Section *1.4*). The putative function of *AtAUX1* (Section *1.4.6*) is that of a cellular auxin influx carrier, possibly, as described by the chemiosmotic hypothesis, in which the substrate affinities for plasma-membrane localised influx/efflux carriers differ between auxins (Section *1.4.5*). This study examined the hypothesis that transformation with the *AtAUX1* gene would enhance the delivery of the root-inducing signal to improve the adventitious rooting capacity of *P. padus*, a model hardwood tree, which is rooting recalcitrant and more or less obligate on exogenous auxin for this process.

7.1.1 Chapter aims

Initial experiments determined optimal conditions for the rooting of wild-type shoots. Due to the lack of transformed non-*35S::AtAUX1* transgenic control plants, wild-type plants are used as controls in the rooting experiments presented in this chapter. Hence, evidence suggesting that the rooting phenotype of (putative) *35S::AtAUX1* transgenic *P. padus* shoots does not result from the process of adventitious shoot regeneration is also presented. The efficacy of auxins, postulated to differ in their mode of cellular influx/efflux, on the rooting capacities of wild-type and (putative) *35S::AtAUX1* transgenic *P. padus* shoot lines, produced as described previously (Chapter *6*), are reported.

7.2 Materials and Methods

7.2.1 Plant materials

Unless stated otherwise, fastigiate *Prunus padus* shoot cultures were initiated, maintained, excised, and transferred to rooting medium as described previously (Chapter *2*). The *35S::AtAUX1* transgenic *P. padus* shoot lines were produced as described in Chapter *6*.

For experiment *7.3*, 24 leaves were excised from *P. padus* shoot cultures and transferred singly to CCPs containing REM2 (Chapter *6*). Explants were subcultured to fresh REM2 after 28 d of culture, and after a further 28 d, 6 cultures were selected arbitrarily from 23 that had produced adventitious shoots. A single shoot, from each culture was transferred to SHM3 and propagated over a further three subcultures to produce sufficient shoot to test rooting.

7.2.2 Rooting experiments

Excised shoots were inoculated (4 per CCP) into semi-solid agar only **ro**ot-induction-**m**edium 3 (ROM3) with or without auxin(s), in accordance with individual experiments:

Experiment 7.1: Wild-type shoots were inoculated into ROM3 with 14.7 μM IBA (indole-3-butyric acid).

Experiment 7.2: Wild-type shoots were inoculated into ROM3 without or with 0.46, 0.92, 1.84, 3.68, 7.35, or 14.7 μM IBA.

Experiment 7.3: Wild-type shoots from the non-regenerated parental and six adventitiously regenerated shoot culture lines were inoculated into ROM3 supplemented with 14.7 μM IBA.

Experiment 7.4: Shoots from wild-type and 6 independent transgenic *P. padus* culture lines were inoculated into ROM3 without or with 14.7 μM IBA.

Experiment 7.5: Wild-type and transgenic shoot culture line-4 shoots were inoculated into ROM3 supplemented with either 3.69, 7.38, 14.76, 29.52 or 59.04 μM auxin; within four independent experiments. The auxins IBA, 1-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) were studied.

Experiment 7.6: Shoots from wild-type and transgenic culture line-4, were inoculated into ROM3 supplemented with either 7.35, 29.52, 35.42, 41.38, 47.23, 53.14 or 59.04 μM NAA (concentration range II).

Experiment 7.7: Shoots from wild-type and 6 independent transgenic shoot culture lines were inoculated into ROM3 supplemented with either 14.7 or 59.04 μM IBA.

In each experiment, after 3 d of culture on ROM3, shoots were transferred to root-development-medium 4 [ROM4; consisting of growth-regulator-free DKW medium (Driver and Kuniyki, 1984) with 166.5 mM fructose] for a further 25 d of culture. However, in experiment *7.1*, ROM4 was modified by using growth-regulator-free WP medium (Lloyd and McCown, 1981) instead of DKW medium, and both media were replicated with the inclusion of 1 mM 1,3,5–trihydroxybenzene (phloroglucinol).

7.2.3 Experimental designs

Rooting experiments contained 20 shoots per treatment, were randomised in blocks, containing one CCP per treatment and repeated twice.

7.2.4 Statistical analyses

Statistical analyses were carried out with Genstat V software (Genstat 5 Committee, 1993). Generalised linear models (McCullagh and Nelder, 1989) with binomial error and the logit link function were used to compare the proportions of shoots that rooted. To compare the number of roots per rooted shoot, generalised linear models with positive poisson error and a link function of log (mean -1) were used (Ridout and Demetrios, 1992). The threshold for statistical significance was taken to be the P=0.05 probability level.

7.3 Results

7.3.1 *Experiment 7.1: Effects of medium and phloroglucinol on rooting and quality of wild-type shoots*

Replicate experiments did not differ significantly (P=0.05) in response to treatments, therefore, data were pooled for further analysis. Supplementing ROM4 with 1 mM phloroglucinol or the substitution of growth-regulator-free DKW-medium with WP-medium had no significant effects on the proportions of wild-type shoots that rooted or the number of roots per rooted shoot (both P=0.05). Over all treatments 94 ± 2.4 % of shoots rooted with 3.18 ± 0.53 roots per rooted shoot. Overall, a visual inspection of the rooted plants implied that there were no significant differences in the quality of rooted plants from the various treatments. Consequently, in subsequent experiments, growth-regulator-free DKW-medium was used in ROM4 without phloroglucinol.

7.3.2 *Experiment 7.2: Effect of IBA concentration on the rooting of wild-type shoots*

Replicate experiments did not differ significantly (P=0.05) in response to IBA concentration, so data were pooled for further analysis. The proportions of wild-type shoots that rooted on ROM3 increased with IBA concentration (P<0.001; Table *7a*), as did number of roots per rooted shoot (P<0.001).

Table 7a. Proportions (± s.e.) of wild-type *P. padus* shoots that produced adventitious roots, and number of roots per rooted shoot (± s.e.) after culture on ROM3 for 3 d without or with various concentrations of IBA, followed by 25 d of culture on ROM4

Concentration (μM)	Proportions of shoots with roots (%)	Roots per rooted shoot
0.00	10 ±4.7	1.50
0.46	10 ±4.7	1.70
0.92	17.5 ±6.0	1.45
1.84	32.5 ±7.4	1.80
3.68	50.0 ±7.9	2.25
7.35	72.5 ±7.1	2.70
14.7	95 ±3.4	2.70

Values were derived from two experiments of 20 replicates per treatment

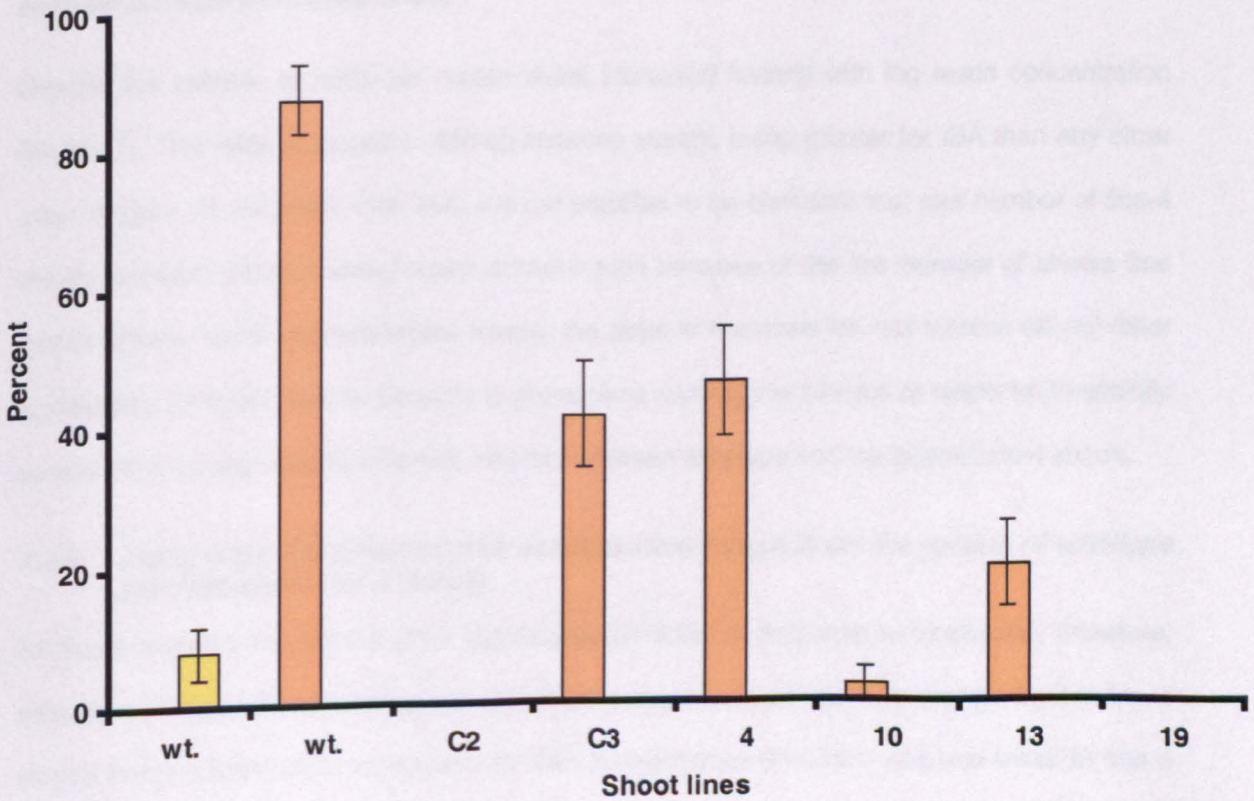
7.3.3 *Experiment 7.3: Effect of adventitious shoot regeneration on the subsequent rooting of wild-type shoots*

There were no significant (P=0.05) differences in the proportions of *P. padus* shoots from a non-regenerated (parental) and six regenerated shoot lines that rooted, the pooled means (± se.) of which were 90 ± 4.7 % (parental) and 94 ± 1.6 % (regenerated). Similarly, there were no significant (P=0.05) differences in the number of roots produced per rooted shoot, with pooled means (± se.) of 3.47 ± 0.29 and 3.30 ± 1.11, respectively.

7.3.4 *Experiment 7.4: Effect of 14.7 μM IBA on the rooting of shoots from wild-type and six transgenic shoot lines*

Between replicate experiments, individual shoot lines did not differ significantly (P=0.05) in their response to IBA, therefore, data were pooled for further analysis. The proportions of wild-type shoots that rooted were significantly greater where root-induction-medium was supplemented with 14.7 μM IBA than without auxin (P<0.001; Figure *7a*). No *35S::AtAUX1* transgenic shoots rooted on medium without IBA (data not shown on graph). Overall, on medium with auxin, the proportion of transgenic shoots that rooted was significantly lower than that of wild-type shoots (P<0.001). There were no significant differences in the proportions of transgenic line-C3 and 4 shoots that rooted (P=0.05), both of which rooted significantly less well than wild-type shoots (P<0.01), but better than line-13 (P<0.05), C2, 10 and 19 (all P<0.001) shoots.

Figure 7a. Proportions of wild-type (wt.) and *35S::AtAUX1* transgenic *P. padus* shoots that produced adventitious roots after culture on ROM3 for 3 d without (□) or with (■)14.7μM IBA, followed by 25 d of culture on ROM4. Data for transgenic shoots without auxin are not shown in this graph as none rooted (bars = ± s.e.)



Values were derived from two experiments of 20 replicates per treatment

7.3.5 *Experiment 7.5: Effect of auxin type and concentration on the rooting of wild-type and transgenic line-4 shoots*

Proportions of shoots that rooted

Replicate experiments did not differ significantly (P=0.05) in their response to specific auxins, therefore, data were pooled for further analysis. For the auxins IAA, IBA and NAA, the proportions of wild-type and transgenic line-4 shoots that rooted increased linearly with log auxin concentration and logit proportion (all P<0.001; Figure *7b* overleaf). However, with 2,4-D the proportions of wild-type and transgenic line-4 shoots that rooted peaked at lower concentrations, displaying a significant (P<0.001) curvature to response. Between auxins, the rates at which rooting increased with concentration varied significantly (P<0.001). Within auxins, there were no significant (P=0.05) differences between wild-type and transgenic line-4 shoots in the kinetics of response to increasing auxin concentration. However, there was a significant (all P<0.001) shift in response within individual auxins, with transgenic line-4 shoots having a lower proportion of rooted shoots at any given concentration (P<0.001).

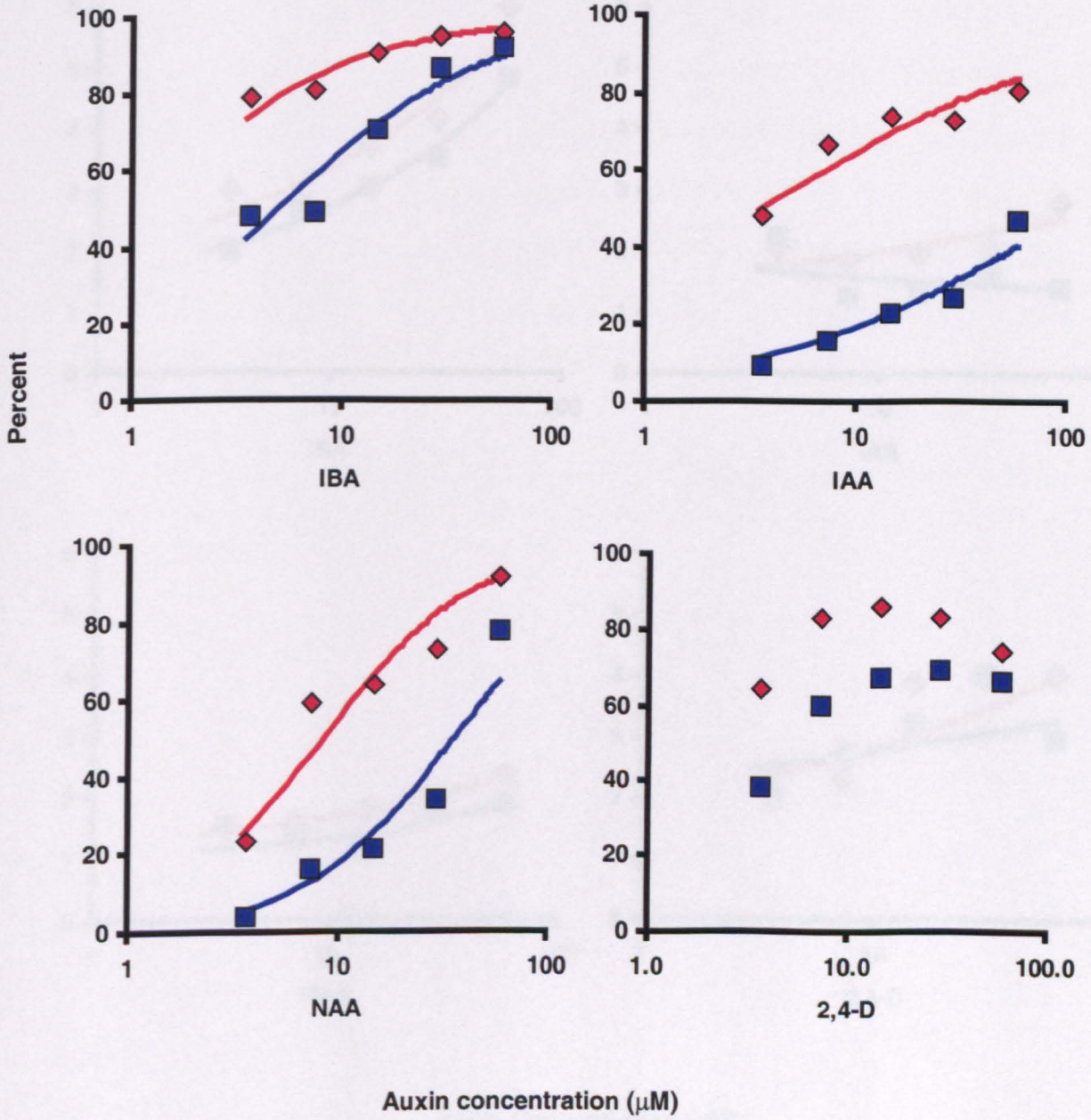
Number of roots per rooted shoot

Overall, the number of roots per rooted shoot increased linearly with log auxin concentration (P<0.001). The rates of increase differed between auxins, being greater for IBA than any other auxin (Figure *7c*, overleaf). With IAA, it is not possible to be confident that root number of line-4 shoots declined with increasing auxin concentration because of the low number of shoots that rooted at lower auxin concentrations. Hence, the slope of response for root number did not differ significantly (P=0.05) from 0. Similarly to proportions rooting, the kinetics of response to specific auxins were not significantly (P=0.05) different between wild-type and transgenic line-4 shoots.

7.3.6 *Experiment 7.6: Effect of NAA concentration (range II) on the rooting of wild-type and transgenic line-4 shoots*

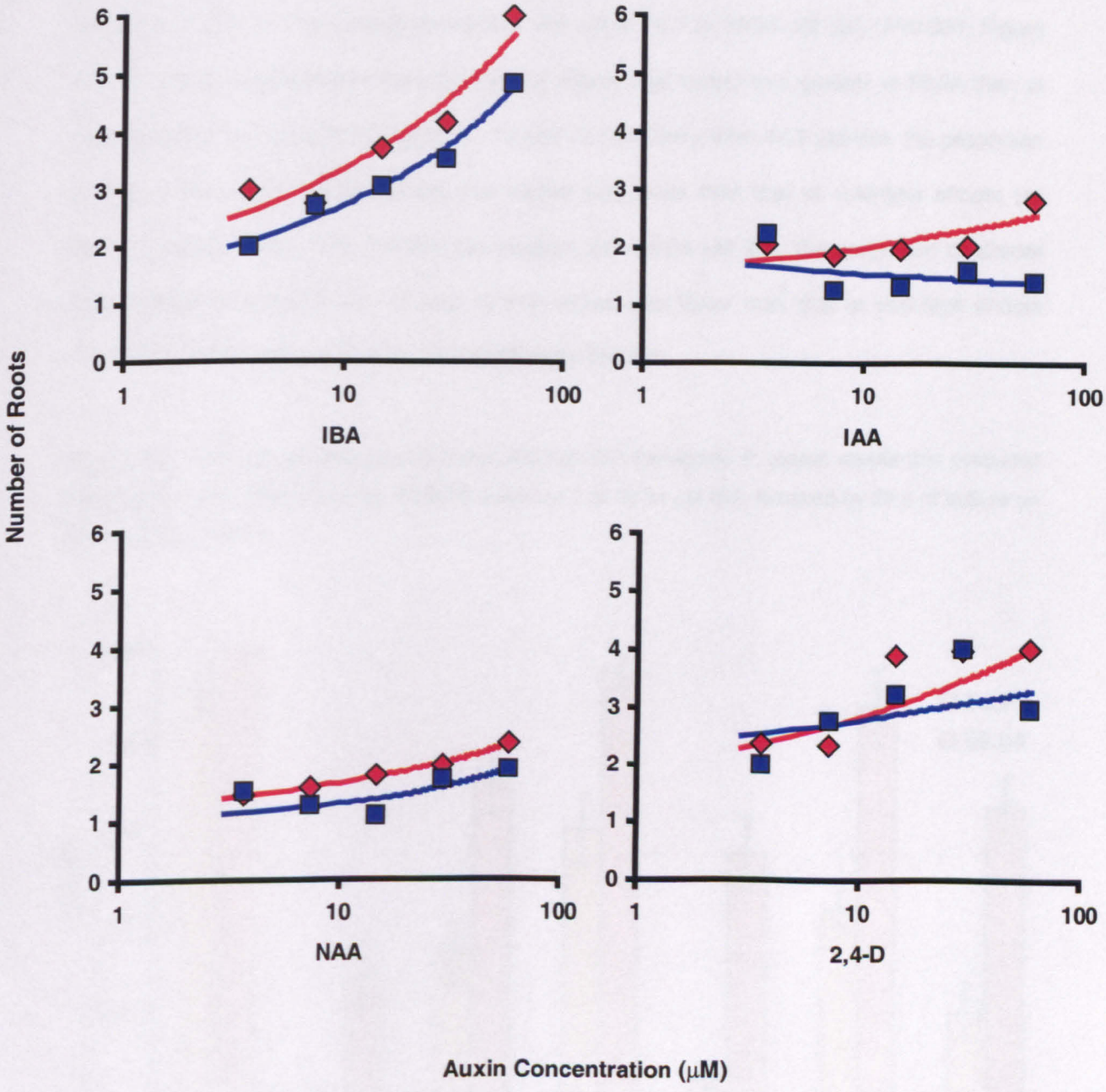
Replicate experiments did not differ significantly (P=0.05) in response to treatments, therefore, data were pooled for further analysis. Overall, the proportions of wild-type and transgenic line-4 shoots that produced roots increased with NAA concentration (P<0.001) and was lower for line-4 than wild-type shoots at a given concentration (data not shown). There was no significant (P=0.05) lack of fit from a linear model for the increase in rooting capacity with NAA concentration.

Figure 7b. Proportions of wild-type (diamonds; predicted values =) and *35S::AtAUX1* transgenic line-4 (squares; predicted values =) *P. padus* shoots that produced adventitious roots after culture on ROM3 for 3 d with different auxins followed by 25 d of culture on ROM4



Values were derived for each auxin from two experiments with 20 replicates per treatment

Figure 7c. Number of roots per rooted shoot on wild-type (diamonds; predicted values = ▬) and *35S::AtAUX1* transgenic line-4 (squares; predicted values = ▬) *P. padus* shoots after culture on ROM3 for 3 d with different auxins followed by 25 d of culture on ROM4



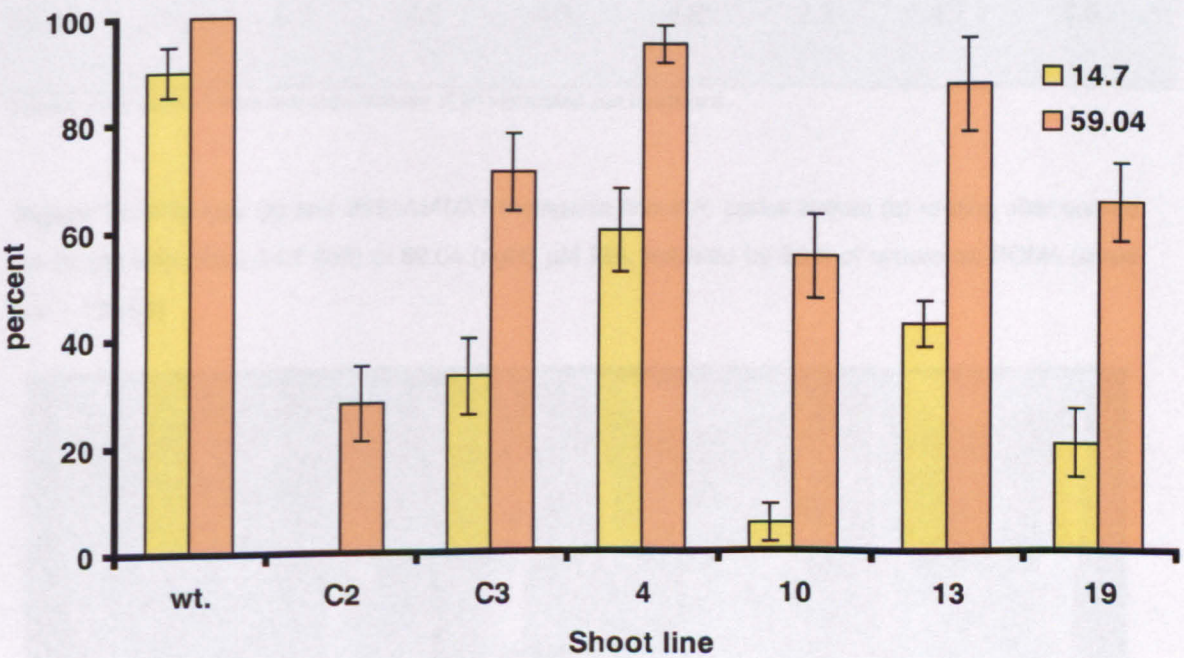
Values were derived for each auxin from two experiments with 20 replicates per treatment

7.3.7 *Experiment 7.7: Effects of 14.7 and 59.04 μM IBA on the rooting of shoots from wild-type and six 35S::AtAUX1 transgenic shoot lines*

Proportions of shoots that rooted

Responses to treatments were not significantly (P=0.05) different between experiments so data were pooled for further analysis. There was no significant difference in the proportion of wild-type shoots that rooted on root-induction-medium with either 14.7 or 59.04 μM IBA (P<0.001; Figure *7d*). For each transgenic line the proportion of shoots that rooted was greater at 59.04 than at 14.7 μM IBA, C3 and 4 (P<0.01), C2, 10, 13 and 19 (P<0.001). With 14.7 μM IBA, the proportion of shoots from each transgenic line that rooted was lower than that of wild-type shoots (all P<0.001 except, line-4 with P<0.01). On medium with 59.04 μM IBA, the proportion of shoots from transgenic lines-C2, C3, 10 and 19 that rooted was lower than that of wild-type shoots (P<0.001), but lines-4 and 13 were not significantly different.

Figure 7d. Proportion of wild-type (wt.) and *35S::AtAUX1* transgenic *P. padus* shoots that produced adventitious roots after culture on ROM3 3 d with 14.7 or 59.04 μM IBA, followed by 25 d of culture on ROM4 (bars = ± s.e.)



Values were derived from two experiments of 20 shoots per treatment

Number of roots per rooted shoot

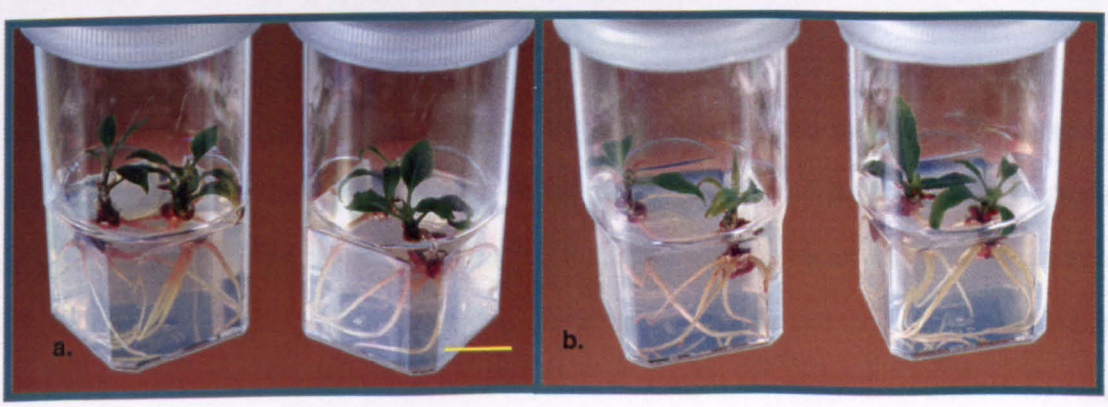
Overall, the number of roots per rooted shoot were greater on 59.04 than 14.7 μM IBA (P<0.001; Table *7b*) and transgenic shoots, at both auxin concentrations, had fewer roots than wild-type shoots (P<0.001). Line-10 shoots produced fewer roots than shoots from any other line. The gross morphology of wild-type and transgenic rooted shoots appeared similar (Figure *7e*). However, transgenic shoots were extremely difficult to transfer *ex vitro*. Shoots from lines-C3 and 4 (17 and 24, respectively) were eventually transferred *ex vitro*, but these failed to survive over-winter within a unheated glasshouse.

Table 7b. Mean number of adventitious roots per rooted shoot of wild-type (wt.) and *35S::AtAUX1* transgenic *P. padus* shoots after culture for 3 d on ROM3 with either 14.7 or 59.04 μM IBA, followed by 25 d of culture on ROM4

IBA concentration (μM)	Shoot line						
	Wt.	C2	C3	4	10	13	19
14.7	3.4	1.7	2.1	1.8	1.0	1.7	1.2
59.04	5.9	4.0	4.3	4.2	2.3	4.7	4.0

Values were derived from two experiments of 20 replicates per treatment

Figure 7e. Wild-type (a) and *35S::AtAUX1* transgenic line-4 *P. padus* shoots (b) rooting after culture on ROM3 with either 14.7 (left) or 59.04 (right) μM IBA, followed by 25 d of culture on ROM4 (scale bar = 10 mm)



7.4 Discussion

Optimising the quality of wild-type rooted shoots

At the onset of this study, rooting capacity/quality of wild-type shoots was characterised. Phloroglucinol (PG) can improve rooting of *Prunus in vitro* [Hammatt and Grant, (1993), Hammatt (1993b)], possibly, by interacting with auxin metabolism directly or by maintaining tissue redox potential in the reduced state (Wilson and Van Staden, 1990). Hammatt (1993a) reported that PG improved the rooting of *P. padus* shoots taken from sub-optimal shoot proliferation medium, but shoots from an optimised medium (thesis medium abbreviation SHM3; Section *2.3.2*) were not assessed by the author. Additionally, a semi-solid agar/auxin-only root induction medium promoted optimal rooting (i.e., ROM3), but low ionic composition media were not tested. These omissions were investigated in this study. Overall, rooting performance of *P. padus* was superior to that reported previously, but rooting capacities and visual shoot quality did not differ significantly between treatments using ROM3 with transfer after 3 d to ROM4 containing either DKW or WP-medium ± PG. Perhaps, this result derives from better shoot quality, and/or a higher 'apparent rejuvenation' status of this shoot line, factors reported to negate the promotory effects of PG [Hammatt and Grant, (1993), Hammatt (1993a/b)]. Hence, in subsequent experiments, growth-regulator-free DKW-medium without PG (i.e., ROM4) was used.

Determining an appropriate IBA concentration for testing rooting hypotheses

Overall objectives for this chapter were initially to test the hypothesis that *35S::AtAUX1* transgenic shoots would root more easily than wild-type shoots. Hence, a sub-optimal IBA concentration was sought that would give a control value sufficient to root wild-type shoots, but providing scope to test this idea with transgenic shoots.

Data from experiment *7.2* indicated that exogenous auxin was required for efficient rooting of wild-type shoots, although, a few shoots rooted on auxin-free medium. Proportions of shoots that rooted, and number of roots per rooted shoot, increased with progressively increasing auxin concentration. An appropriate concentration to test an unbiased hypothesis would have been 3.68 μM IBA where 50 ± 7.9 % rooted. However, surprising, preliminary results

(data not shown) with the rooting capacity of *35S::AtAUX1* transgenic shoots were to change this strategy.

Influence of adventitious shoot regeneration on subsequent rooting performance

Problems associated with confirming the status of putative *35S::AtAUX1* transgenic *P. padus* cultures by molecular analyses were discussed previously (Section *6.4.4*). The lack of an appropriate transformed control (i.e., transformed without *AtAUX1*) was of concern in this chapter as confidence that the observed rooting phenotype resulted exclusively from *35S::AtAUX1* expression was reduced. Consequently, the rooting capacities of putative transgenic shoot lines were compared against that of wild-type shoots.

Somaclonal variation (i.e., phenotypic variation amongst plants regenerated from callus, as a result of morphogenesis having been initiated from (a) genetically aberrant cell(s)) has been observed in a range of species (George, 1993). Adventitious shoot regeneration from *P. padus* leaves is predominantly indirect, and obligate on a callus phase when under selection pressure from kanamycin-mono-sulphate; hence, making it potentially liable to somaclonal variation. The genetic stability of *P. padus* callus is unknown; this can be influenced by many factors, such as, prolonged culture, genotype (Flashman, 1982) and ploidy, particularly aneuplody. However, somaclonal variation is considered (Novak, 1980) less likely to occur with diploid cells.

To be confident that the shoot regeneration process did not influence the subsequent adventitious rooting capacity of regenerated shoots, the hypothesis that somaclonal variation might explain altered rooting response(s) in transgenic lines was also investigated. Data from six independently regenerated shoots showed no significant differences in rooting capacities. No other obvious aberrant phenotypic variation were observed in the regenerated shoots from that of the control (wild-type). Thus, there is a low likelihood that any modified rooting phenotype seen in *35S::AtAUX1* shoots could be ascribed to the adventitious shoot regeneration process *per se*, but it does not entirely discount any effects associated with T-DNA insertion.

Rooting of the six At35S::AUX1 transgenic Prunus padus shoot lines

A combination of factors, e.g., the apparent reduced viability of putative transgenic callus and a deficiency in shoot regeneration capacity from this callus, previously discussed in Chapter *6*,

raised the possibility that *35S::AtAUX1* transgenic shoots were hypersensitive to auxin. Hence, I made the decision to test rooting on medium with and without auxin.

That *35S::AtAUX1* transgenic shoots would have higher rooting than wild-type shoots on medium with or without exogenous auxin was tested in experiment *7.4*. Proportions of wild-type shoots that rooted on medium with 14.7 μM IBA (87.5 ± 4.7 %) and without (7.7 ± 3.8 %) IBA were consistent with previous experiments. However, no transgenic shoots (total tested = 240) rooted on medium without auxin and rooting of all six transgenic lines was significantly less on medium with auxin than that of wild-type shoots on similar medium. Furthermore, the proportions of shoots that rooted differed significantly between transgenic shoot lines, most notably, shoots from lines C2 and 19 did not root, and with line-10 a minimal proportion of 2.5 ± 2.4 % rooted on medium with auxin.

These results support the view that somaclonal variation appears improbable, since on each occasion a deleterious, albeit by varying degrees, rooting phenotype was observed in all six independently transformed shoot lines. However, there are several possible explanations that may explain the differences in rooting capacities:

1. Constitutive transgene expression promoted disruption of directed auxin transport: Possibly, expression of *AtAUX1*, under the constitutive *CaMV 35S* promoter, resulted in the recruitment of cells not ordinarily involved in auxin transport. Thus, disruption of the auxin transport mechanism may have ensued, the rooting signal having become disordered and/or abated as non-specific cells entrap auxin. Evidence suggesting the probable inhibitory consequences on adventitious rooting of disrupted basipetal auxin transport have been discussed previously (Section *1.4.10*).

2. Transgene-induced gene silencing: Epigenetic, i.e., mitotically and/or meiotically heritable changes in the function of a gene without changes to the DNA sequence, can occur at either the transcriptional or post-transcriptional level. Transgenes can undergo silencing after integration into the host genome, as can the genes of the host as a consequence of the presence of homologous transgenes [Meyer and Saedler (1996), Vaucheret *et al.* (1999), Kooter *et al.* (1999)].

Transcriptional silencing occurs mainly when multiple repeats of the transgene are inserted and correlates with chromatin condensation and hypermethylation. Nevertheless,

silencing of single copies can occur if inserted into hypermethylated loci or the transgene sequence is recognised as foreign to the surrounding genomic sequence (Gelvin, 1998).

Post-transcription gene silencing predominantly results from the production of abnormally high levels of RNA transcription, under expression from strong promoters (e.g., *CaMV 35S*), and the subsequent cellular mechanisms involved in degradation of this RNA. Possibly, activation of these mechanisms occurs as a result of transcription breaching a putative threshold level (Dehio and Schell, 1994), although, total transcription levels may not always be elevated, suggesting a sub-set of RNA or other factors are involved (English *et al.*, 1996).

Co-suppression, the silencing of transgene(s) and homologous host gene(s) may occur in a reciprocal and synergistic interaction (Napoli *et al.*, 1990), which has been correlated with the level of transgene product, and expression under a strong promoter (Vaucheret *et al.*, 1999).

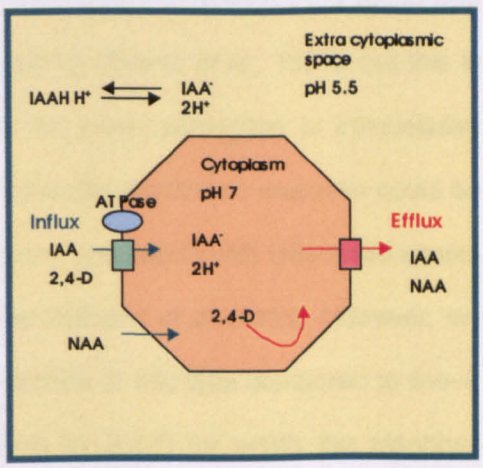
3. Disruption of host gene(s) controlling rhizogenesis: The number of copies of a transgene that integrate into the genome of a transformed plant and the position of the site of integration cannot be predicted (Section *6.1.3*). However, considering the number of potential sites, the probability that T-DNA integration would have resulted in disruption of genes specifically involved in rhizogenesis in six transgenic lines appears remote.

It is possible that one or a combination of these factors may be acting to produce the rooting phenotype observed in *35S::AtAUX1* transgenic shoots. From these possible hypotheses I considered the most probable, and therefore worthy of testing, are disruption of directed auxin flux due to constitutive transgene expression and co-suppression. Both hypotheses suggest inhibition of auxin transport, predicted to reduce rooting capacity (section *1.4.10*), and the expression of the strong CaMV 35S constitutive promoter fits with this theme. Furthermore, the homologue of *AtAUX1*, *CHAX1*, was isolated in our laboratory (Hand *et al.*; unpublished data) on the basis of the high degree of homology between these genes, and it is plausible that homology may exist between *AtAUX1* and other members of this gene family in cherry.

Effect of increasing auxin concentrations to saturate the influx carrier

The substrate affinity of different auxins for carriers varies (Figure *7f*), as does auxin plasma-membrane permeability. Thus, NAA can enter the cell by passive diffusion, whereas, IAA and 2,4-D are actively influxed. Intracellular auxin concentration is regulated by the efflux carrier for NAA, whereas 2,4-D is regulated by influx, and IAA by both mechanisms (Delbarre, 1996). Thus far, the mechanism by which IBA is transported across the plasma membrane is unknown.

Figure 7f. Putative model of auxin flux across the plasma-membrane



The hypothesis tested in this study was whether rooting capacity improved with increasing auxin concentration and whether the rooting response to auxin differed between auxins postulated to be substrates for the putative influx carrier (IAA and 2,4-D) or that enter the cell by passive diffusion (NAA).

The proportions of shoots that rooted increased with increasing auxin concentration both for wild-type and transgenic line-4 shoots. Overall, irrespective of auxin type, the proportions of line-4 shoots that rooted were lower than wild-type shoots. At the midpoint concentration of 14.7 μM, the most effective auxins at promoting rooting of line-4 shoots were IBA and 2,4-D. Since IAA was less effective both in line-4 and wild-type shoots at this concentration, the difference in response may reflect relative differences in metabolism between auxins. Indeed 2,4-D not being metabolised, or poorly metabolised, may explain why its response differed from that of the other auxins. This result is also consistent with 2,4-D not being effluxed actively; either way, the data suggest that the concentration of this auxin became phytotoxic. From a practical point of view, the proportions of line-4 and wild-type shoots that rooted were comparable at 59.04 μM IBA. This result is interesting, since IBA is a commonly used auxin in tissue culture, and was not assessed in the study by Delbarre *et al.* (1996). IBA is structurally similar to IAA, and perhaps they are both fluxed across the cellular membrane in an analogous manner. Of particular significance NAA appears to be behaving as if it were a substrate for the influx carrier.

Overall, the pattern observed in number of roots per rooted shoot was similar to that of proportions rooting. However, with IAA there was no evidence to suggest that mean root number per rooted line-4 shoot either increased or declined with increasing IAA concentration. Furthermore, data from 2,4-D did not show significant decline in response with increasing concentration of auxin.

Consistent with *AtAUX1* having a role in auxin influx, auxin-induced rooting responses take twice as long for the *Ataux1* mutant than wild-type plants (Evans *et al.*, 1994), but this is conditional upon the assumption that at least one site for auxin perception is intracellular. Additionally, Yamamoto and Yamamoto (1998) observed that the gravitropic response could be restored in *Ataux1* roots by NAA, but not IAA or 2,4-D. This is compatible with differential uptake of auxins by tobacco suspension-cultured cells reported by Delbarre *et al.* (1996). However, my results contradict this conclusion because the response kinetics of wild-type compared to line-4, particularly when compared to IAA and NAA, rather than for 2,4-D for which the response kinetics were clearly different over the concentration range assessed, did not differ (this conclusion is notwithstanding the possibility that at higher concentrations of auxin similar declines in rooting response to that seen with 2,4-D may occur). However, it is unknown to what extent NAA uptake is rapidly alleviated by rapid efflux, as was shown to occur in tobacco cell cultures (Delbarre *et al.*, 1996), or how auxin conjugation is influenced by transgene expression.

The current state of knowledge on auxin transport is insufficient to allow a full interpretation of these results. The hypothesis that auxin may have been taken up by cells acting as sinks to entrap auxin cannot be confirmed or refuted by the apparent difference between rooting responses to different auxins predicted by Delbarre's model of the auxin uptake carrier (hypothesised to be *AtAUX1*) and the efflux carrier, and the results obtained with *P. padus* shoots transformed with constitutively expressed *AtAUX1*. There remains the question, is *AtAUX1* really an auxin influx carrier?

Checking that NAA response was linear

Experiment *7.6* tested the hypothesis that the proportions of wild-type and line-4 shoots that rooted with increasing NAA concentration could be modelled adequately by a linear model. This was because there appeared to be some evidence, thought not statistically significant, that there

was a concentration threshold in response in experiment *7.5*, which may have indicated involvement of active uptake. This would have been unusual since the influx of NAA is predicted to be passive. However, data suggest there was no lack of fit to a linear model. Hence, the hypothesis was accepted and provided greater confidence that the response to NAA in experiment *7.5* was indeed linear, and therefore, unlikely that a threshold response existed.

All six At35S::AUX1 transgenic P. padus shoot lines root on high IBA concentration

Experiment *7.6* added confidence to the results of previous experiments where only transgenic line-4 shoots had been tested, and checked the model for auxin saturation established in experiments *7.5*.

Data from experiment *7.6* indicated that the proportions of transgenic shoots that rooted on medium with 14.7 μM IBA were all significantly lower than wild-type shoots on similar medium, and the overall pattern of rooting capacities was comparable with data from experiments *7.4*, the most notable exception being that line-19 shoots on this occasion rooted. On medium with 59.04 μM IBA the proportions of shoots that rooted increased for all transgenic lines. It was now possible to root transgenic shoot lines C2, C10 and 19, and the proportions of transgenic lines-4 and 13 that rooted were not significantly different from that of wild-type shoots. Similar patterns of response were recorded for number of roots per rooted shoot. For wild-type and each transgenic shoot line, significantly more roots were produced at the higher auxin concentration. However, at both concentrations, wild-type shoots had more roots per shoot than for any transgenic line. Significantly fewer roots were produced on line-10 shoots than any other transgenic line.

These results indicate that the rooting capacity of all transgenic shoots, at least with IBA, can be restored by increasing exogenous auxin. It is possible that increasing exogenous auxin concentration may further improve the rooting capacity of transgenic lines-C2 and 10. However, it is possible that auxin concentration may become phytotoxic for these lines before rooting would be resorted to levels comparable to that of wild-type shoots.

Conclusions

Adventitious rooting of all six *35S::AtAUX1* transgenic shoot lines was lower than that of wild-type shoots, and rooting profiles differed between transgenic shoot lines, but were generally

consistent throughout this study for individual lines. Data suggest the reduction in rooting was not due to auxin hypersensitivity or inhibition of root primordia development: transgenic shoots failed to root on auxin-free medium and increasing auxin concentration improved rooting. Rooting capacity was increased for all transgenic lines if more auxin was applied.

Results suggest that expressing the *AtAUX1* gene under the *CaMV 35S* promoter may have been detrimental to the adventitious rooting of transgenic shoots, possibly, inhibition of auxin transport and/or co-suppression may have resulted. However, for any of these hypothesis to be tested would require further molecular evidence (e.g., Southern and RT-PCR analysis) which currently has been unsuccessful (Section *6.4.4*).

To improve rooting, it may be necessary to limit expression temporally by placing the gene under the control of an inducible promoter [e.g., Dexamthasone; Aoyama and Chua (1997)]. Alternatively, expression could be focused in particular target cells/tissues [e.g., *rolB and rolC* promoters which have been found to be predominantly expressed in pericycle cells which serve as root initials in transgenic hybrid aspen; Nilsson *et al.* (1997)]. Additionally, these strategies may be required to achieve transformation, as discussed previously (Section *6.4.5*). It would also be desirable to have more transgenic shoot lines than the six produced in this study, and to have a transformed control (without *AtAUX1*) to increase confidence in the data.

An alternative strategy may be to use a different transgene. Potential candidate genes may include:

SUR2: This has been cloned and encodes a CYP83B1 protein, a member of the P450 dependent mono-oxygenases. *SUR2* expression is unregulated in the *sur1* mutant (King *et al.*, 1995), and induced by exogenous auxin in the wild-type. However, expression of the gene would require tight regulation.

AXR1: The product of this gene is postulated to be involved in the early stages of auxin signal perception or transduction (Leyser *et al.*, 1993). Loss of function in the *aux1* mutant results in reduced auxin sensitivity.

Regulation of these alternative genes may promote the availability of free auxin and enhance sensitivity, respectively. However, they may require similar targeted and/or controlled expression to be effective. There are many other genes that may be suitable to investigating

adventitious rooting by molecular genetic approach, an interesting example is the *RAC* gene (Muller *et al.*, 1985). The *rac* mutant is resistant to auxin and appears to have a block in the pathway to adventitious root formation beyond that of auxin perception (as cell division in phloem and inner cortical parenchyma cells occurs). Thus, study of this mutant, which appears to have a defect in rooting competence, in tandem with others where competence is enhanced, may provide valuable insights into the overall process [for a general overviews of the *Arabidopsis thaliana* auxin mutants see reviews by Hobbie and Estelle (1994), Walden and Lubenow (1996), and Leyser (1997)].

8. General Discussion

Two cherry species (*Prunus avium* and *P. padus*) were used as models in this study to investigate the physiological and genetic manipulation of adventitious rooting.

The study encompassed three primary objectives:

- Determining the relative contributions components of the micropropagation process had on the rate at which adventitious rooting increased progressively with time in culture, a process which is postulated to result from the 'apparent rejuvenation' of mature tissues.
- Evaluating effects of gibberellins, with predicted structure-activity, and analogy to those found in abundance in the juvenile phase, on maintaining and/or promoting adventitious rooting of mature and *ex vitro* trees.
- Investigating the effects on adventitious rooting of constitutive expression of the putative auxin uptake carrier from *Arabidopsis thaliana AtAUX1* in *P. padus*. However, this required additional objectives related to developing the required shoot regeneration and transformation methodology.

An investigation of the relative contributions of time in culture, and number of subcultures, to the progressive promotion of rooting competence during micropropagation suggest that time spent in culture was the predominant factor leading to improved rooting, and not subculture frequency *per se*, as had been suggested (but not proven experimentally) previously [Webster and Jones (1989), Noiton *et al.* (1992)]. Observations that rooting improved in cultures kept at 4 °C led to fluence and temperature being investigated. Overall, the data suggest that subculture interval and fluence were not factors determining the rate at which rooting improved with time in culture at 4 °C, implying that temperature in the 84 d material, or an unidentified factor (at 24 °C) was involved.

Rooting performance, of non-clonal shoots, increased rapidly, in the period spanning the introduction of shoots *in vitro* to production of proliferating clonal shoot lines, a period where putative markers of juvenility [Hand *et al.* (unpublished data), Oliveira and Browning (1993),

Noiton *et al.* (1992)] have been observed to change rapidly. Preliminary data suggests competence to acquire enhanced rooting may differ between axial and axillary shoots. From a practical point of view, it is pertinent to ask how short can we make the period spent *in vitro* and still be able to achieve improved rooting *ex vitro.*

Apparently-rejuvenated *ex vitro* and hedged (putatively) mature *P. avium* trees were treated with gibberellins predicted to have a range of structural related activities. GA structure-activity, predicted by the model of Oliveira and Browning (1993), within the constraints of GA concentration used and hedge maturity status, suggest that *ex vitro* trees were behaving as if juvenile which would be consistent with the hypothesis of micropropagation promoting the 'apparent rejuvenation' of mature trees. GA_7 promoted rooting of cuttings from hedged (putatively) mature *P. avium*, but not *ex vitro* trees. This could be explained by the genetic rooting potential (i.e., genetically-determined maximum rooting competence) of cuttings from *ex vitro P. avium* being more likely to be near this maximum than mature hedged trees. Observations of potential silvicultural value include the effect of GA_7, in particular, to promote tree height and extend the active growth season.

The putative function of the *Arabidopsis thaliana AtAUX1* gene is that of a cellular auxin influx carrier, as described by the chemiosmotic hypothesis. Hypotheses based on whether the *AtAUX1* gene would enhance the delivery of the auxin root-inducing signal to improve rooting, of *P. padus*, were tested. However, all six, constitutively expressed, cauliflower Mosaic Virus 35S promoter driven, (*35S::AtAUX1*) transgenic shoot lines had reduced rooting. Data suggest the reduction in rooting was not due to auxin hypersensitivity or inhibition of root primordia development: transgenic shoots failed to root on auxin-free medium and increasing auxin concentration improved rooting. From the possible hypotheses that may explain the reduction in rooting, inhibition of auxin transport as a result of constitutive transgene expression leading to disorganised and/or abated flux or co-suppression are the most likely. The rooting response to auxin differed between auxins postulated to be substrates for the putative influx carrier (IAA and 2,4-D) or that enter the cell by passive diffusion (NAA). Most significant was that NAA appeared to be acting as if it were a substrate for the influx carrier. This apparent contradiction could not be resolved.

Developing methodology for an investigation of adventitious rooting via a molecular genetic approach

Methodology to regenerate adventitious shoots from *P. avium* leaf explants was developed further in this study. The basic regeneration protocol published by Hammatt and Grant (1998) could be applied to a wider range of *P. avium* accessions from British woodlands, but regeneration competence was accession-dependent, as found by Yang and Schmidt (1992) for sweet cherry. Failure to induce adventitious shoot regeneration other than directly from the initial explant limited shoot regeneration competence to a very narrow window of opportunity. Furthermore, relatively low numbers of shoots were produced per explant. Of the factors assessed, in an attempt to increase shoots regenerated per leaf, a low concentration of the surfactant tween-20 was the most promotory supplement.

An attempt to transform *P. avium* failed. The most significant factors being, relatively low shoot regeneration capability, unsuitable selection method, and apparent detrimental effect of the *35S::AtAUX1* expression. The latter factor appeared to be a problem during the recovery of transgenic *P. padus* shoots. Putative transgenic *P. padus* plants were produced by an *Agrobacterium tumefaciens* mediated strategy. That callus and shoot regeneration were inhibited under non-selective conditions (and callus growth unrelated to kanamycin mono-sulphate concentration) suggest that selection with this antibiotic was not the primary problem with *P. padus*, hence, the hypothesis that a defect in the *NPTII* gene would lead to lower transformation/regeneration rates was rejected. Overall, data suggests that the activity of the transgene under a constitutive promoter may be detrimental to transgenesis. The inability to produce non-*AtAUX1* transgenic plants either of *P. padus*, tobacco or *Arabidopsis thaliana* suggests a fault within the plasmid constructs used.

An improved transformation strategy for cherry should be based on the use of 'clean technology'. Use of the *ipt*-based dominant selection of the (MAT) system may be very effective with *P. avium*. The postulated detrimental effects of expression of *AtAUX1* under the *35S CaMV* promoter could be prevented by regulating expression under either tissue-focused promoters (i.e., *rolB* or *rolC*) or the dexamthasone inducible promoter (Aoyama and Chua, 1997).

Evidence [i.e., caulogenesis and organogenesis in the presence of kanamycin mono-sulphate concentrations normally inhibitory to growth, reduced rooting capacity of putative transgenic shoots and an inability to mimic this rooting phenotype using shoots derived from cultures established from adventitiously regenerated wild-type shoots] suggest that *35S::AtAUX1* transgenic *P. padus* cultures were produced. PCR analyses support this conclusion. However, further molecular evidence by Southern blotting, to confirm transgene integration, and either northern or RT-PCR analyses to assess levels of expression would be desirable. Knowledge of the level of transgene expression would also be invaluable in testing many of the hypotheses proposed to explain the rooting phenotype of *35S::AtAUX1* transgenic shoots.

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10. Appendices

10.1 Abbreviations

Table 8a. Chemical abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
4-Cl-IAA	4-chloroindole-3-acetic-acid
ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
$AgNO_3$	silver nitrate
Al_2O_3	aluminium oxide
AVG	aminoethoxyvinylglycine
BA	benzyladenine
CTAB	cetyltrimethylammonium bromide
CTX	cefotaxime sodium
dNPT	2-deoxynucleoside-5-triphosphate
EDTA	ethylenediaminetetraacetic acid
EMS	ethylmethylsulphonate
F68	polyoxyethylene-polyoxypropylene co-polymer
FCCP	carbonylcyanade *p*-trifluoromethoxphenyl-hydrazone
GA	gibberellic acid
GUS	β-glucuronidase
IAA	indole-3-acetic acid
IAM	indole-3-acetamide
IAN	indole-3-acetonitrile
IBA	indole-3-butyric acid
IGP	indole-3-glycerol phosphate
IPA	indole-3-pyruvic acid
KCl	potassium chloride
KM	kanamycin mono-sulphate
$MgCl_2$	magnesium chloride
NaCl	sodium chloride
NaOCl	sodium hypochlorite
NPA	*N*-1-naphtylphthalamic acid
PG	1,3,5-trihydroxybenzene
PVP-40	polyvinylpyrrolidone
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
Tween-20	polyoxethylenesorbitan monolaurate

Table 8b. Culture media abbreviations

Abbreviation	Use	Composition	Reference(s)
SHM1	*Prunus avium* shoot culture initiation medium	Modified MS-medium (Murashige and Skoog, 1962), 4.4 µM BA, 0.29 µM GA_3, 0.49 µM, IBA, 1 mM 1,3,5-trihydroxybenzene, 87.7 mM sucrose	Hammatt and Grant (1993, 1997a)
SHM2	*Prunus avium* shoot culture proliferation medium	Modified MS-medium (Murashige and Skoog, 1962), 2.2 µM BA, 0.49 µM, IBA, 1 mM 1,3,5-trihydroxybenzene, 87.7 mM sucrose	Hammatt and Grant (1993, 1997a)
SHM3	*Prunus padus* shoot culture proliferation medium	DKW medium (Driver and Kuniyki, 1984), 4.4 µM BA, 49 nM IBA and 166.5 mM fructose	Hammatt (1993a)
ARM1	*Arabidopsis* seed germination medium	Modified MS-medium (Murashige and Skoog, 1962) with 29.3 mM sucrose	This thesis
TOM1	Tobacco seed germination and node culture medium	Modified MS-medium (Murashige and Skoog, 1962) with 58.5 mM sucrose	This thesis
ROM1	*Prunus avium* root initiation and development medium	Modified MS-medium (Murashige and Skoog, 1962), 14.4 µM, IBA, 87.7 mM sucrose	Hammatt and Grant (1993, 1997a)
ROM2	*Prunus avium* root initiation and development medium	Modified MS-medium (Murashige and Skoog, 1962), 14.4 µM IBA, 1 mM 1,3,5-trihydroxybenzene, 87.7 mM sucrose	Hammatt and Grant (1993, 1997a)
ROM3	*Prunus padus* root initiation medium	6 gl^{-1} agar only with or without auxin at various concentrations	Hammatt (1993a)
ROM4	*Prunus padus* root development medium	DKW-medium (Driver and Kuniyki, 1984) and 166.5 mM fructose	Hammatt (1993a)
REM1	*Prunus avium* shoot regeneration medium	WP-medium (Lloyd and McCown, 1981) with 0.54 µM NAA, 4.4 µM TDZ and 87.7 mM sucrose	Grant and Hammatt (2000)
REM2	*Prunus padus* shoot regeneration medium	WP-medium (Lloyd and McCown, 1981) with 0.54 µM NAA, 4.4 µM BA and 87.7 mM sucrose	Hammatt (1993a)
REM3	Tobacco shoot regeneration medium	Modified MS-medium (Murashige and Skoog, 1962) with 4.4 µM BA, 1.08 µM NAA and 60.5 µM glucose	This thesis

10.2 List of Tables

10.3 List of Figures

10.4 Publications Derived from Thesis

GRANT, N.J., and HAMMATT, N. (1999). Increased root and shoot production during micropropagation of cherry and apple rootstocks: effect of subculture frequency. Tree Physiol. 19: 899-903.

GRANT, N.J., and HAMMATT, N. (1999). Constitutive ectopic expression of the *Arabidopsis thaliana AUX1* gene in *Prunus padus* reduces rooting competence. Tree Biotechnology 99. Oxford, UK. 11-16 July 1999.

GRANT, N.J., and HAMMATT, N. (2000). Adventitious shoot development from wild cherry (*Prunus avium* L.) leaves. New Forests. (In press).

GRANT, N.J., BENNETT, M.J., and HAMMATT, N. (In progress). Constitutive ectopic expression of the *Arabidopsis thaliana AUX1* gene in *Prunus padus* reduces rooting competence. Target Journal Planta.

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Grau, teurer Freund, ist alle Theorie Und grun des Lebens goldner Baum.

Johann Wolfgang von Goethe 1749-1832